

Development of Molecular Chimeras Targeting the NLRP3 Inflammasome and HDAC6

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List of Abbreviations

α	cooperativity
ΔT_m	difference in protein melting temperature
δ	chemical shift
AIM2	absent in melanoma 2
Ala	alanine
AMC	7-amino-4-methylcoumarin
AR	androgen receptor
arom.	aromatic
ASC	apoptosis-associated speck-like protein containing a CARD
APCI	atmospheric pressure chemical ionization
API	active pharmaceutical ingredient
ATP	adenosine triphosphate
ADP	adenosine diphosphate
aq	aqueous
AUC	area under the curve
BAIB	bis(acetoxy)iodobenzene
<i>BCR-ABL</i>	breakpoint cluster region gene/Abelson proto-oncogene
BIR	baculoviral IAP repeat
BMDM	bone marrow-derived macrophage
Bn	benzyl
Boc	<i>tert</i> -butyloxycarbonyl
Boc ₂ O	di- <i>tert</i> -butyl dicarbonate
BODIPY	boron-dipyrromethene
BRD4	bromodomain 4
bRo5	beyond rule of five
BTC	bis-(trichloromethyl) carbonate, triphosgene

List of Abbreviations

Bu	butyl
calcd	calculated
CAPS	cryopyrin-associated periodic syndrome
CARD	caspase recruitment domain
ca.	circa
Cbz	benzyloxycarbonyl
CD	catalytic domain
CDCl ₃	deuterated chloroform
CDK6	cyclin-dependent kinase 6
Chg	cyclohexylglycine
cIAP1	cellular inhibitor of apoptosis protein 1
cIAP2	cellular inhibitor of apoptosis protein 2
CLR	C-type lectin receptor
CRABP	cellular retinoic acid-binding protein
CRBN	cereblon
CRBN/DDB1ΔB	complex of CRBN with the modified adaptor protein DDB1ΔB
CRID	cytokine release inhibitory drug
cryo-EM	cryogenic electron microscopy
CuAAC	copper(I)-catalyzed azide-alkyne cycloaddition
CIITA	MHC class II transcription activator
d	doublet
dd	doublet of doublets
<i>d</i> ₆	hexadeuterated
DAD	diode-array detection
DAMP	damage-associated molecular pattern
DARTS	drug affinity responsive target stability
DCHA	dicyclohexylamine
DFMO	difluoromethyl-1,3,4-oxadiazole
DIPEA	<i>N,N</i> -diisopropylethylamine, Hünig's base

DMAP	<i>N,N</i> -dimethylpyridin-4-amine
DMF	dimethylformamide
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EDC × HCl	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride
<i>e.g.</i>	for example (<i>exempli gratia</i>)
<i>elogD</i>	experimental distribution coefficient
<i>elogD</i> _{7.4}	experimental distribution coefficient at a physiological <i>pH</i> of 7.4
eq	equivalent
ER	estrogen receptor
ESI	electrospray ionization
Et	ethyl
E1	ubiquitin-activating enzyme
E2	ubiquitin-conjugating enzyme
E3	ubiquitin ligase
<i>et al.</i>	and others (<i>et alii</i>)
FCAS	familial cold autoinflammatory syndrome
FDA	Food and Drug Administration of the United States of America
FOXO1	forkhead box protein O1
GSDMD	gasdermin D
GSDMD-N	gasdermin N
h	hour
HAT	histone acetyltransferase
HATU	1-[Bis(dimethylamino)methylene]-1 <i>H</i> -1,2,3-triazolo[4,5- <i>b</i>]pyridinium 3-oxide hexafluorophosphate
HDAC	histone deacetylase
Hda1	histone deacetylase A1
HD1	helical domain 1
HD2	helical domain 2

HET-E	incompatibility locus protein from <i>Podospora anserina</i>
HIF1 α	hypoxia-inducible factor 1 α
HMDM	human monocyte-derived macrophage
HOBt	<i>N</i> -hydroxybenzotriazole
HPLC	high-performance liquid chromatography
HRMS	high-resolution mass spectrometry
HSP90	heat shock protein 90
HTRF	homogenous time-resolved fluorescence
Hyp	hydroxyproline
<i>i</i>	<i>iso</i>
IAP	inhibitor of apoptosis protein
IBD	inflammatory bowel disease
IC ₅₀	half-maximal inhibitory concentration
<i>i.e.</i>	that is (<i>id est</i>)
IgE	immunoglobulin E
IL	interleukin
IL-1R	interleukin-1 receptor
IMiD	immunomodulatory imide drug
ITC	isothermal titration calorimetry
IRAK1/4	interleukin-1 receptor-associated kinases 1 and 4
<i>J</i>	coupling constant
K_D	kinetic dissociation constant
K_D^{binary}	dissociation constant for binary complex formation
K_D^{ternary}	dissociation constant for ternary complex formation
LC	liquid chromatography
LDH	lactate dehydrogenase
LHS	left-hand side of the VHL/HIF1 α interaction
lit.	literature
LPS	lipopolysaccharide

LRR	leucine-rich repeat
m	multiplet
MBP	maltose-binding protein
MBP-NLRP3	MBP-NLRP3 fusion protein
MDM2	mouse double minute 2 homolog
Me	methyl
min	minute
MNS	3,4-methylenedioxy- β -nitrostyrene
mp	melting point
MS	mass spectrometry
MsCl	methanesulfonyl chloride
MSU	monosodium urate
MTOC	microtubule-organizing center
mtROS	mitochondrial reactive oxygen species
MW	molecular weight
MWS	Muckle-Wells syndrome
MyDD88	myeloid differentiation primary response 88
<i>m/z</i>	mass-to-charge ratio
NACHT	(<u>NA</u> IP, <u>CI</u> ITA, <u>HE</u> T-E and <u>TP</u> 1)
NAD ⁺	oxidized nicotinamide adenine dinucleotide
NAIP	neuronal apoptosis inhibitory protein
NALP3	NACHT, LRR and PYD domains-containing protein 3
nanoDSF	nano differential scanning fluorimetry
NASH	nonalcoholic steatohepatitis
NBD	nucleotide-binding domain
NEK7	NIMA-related kinase 7
NF- κ B	nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
NHS	<i>N</i> -hydroxysuccinimide
NLR	NOD-like receptor

NLRC4	NLR family CARD domain-containing protein 4
NLRP3	nucleotide-binding oligomerization domain, leucine-rich repeat and pyrin domain-containing protein 3
NMPA	Chinese National Medical Products Administration
NMR	nuclear magnetic resonance
No.	number
NOD	nucleotide-binding oligomerization domain
NOMID	neonatal-onset multisystem inflammatory disorder
ox-mtDNA	oxidized mitochondrial DNA
PAMP	pathogen-associated molecular pattern
Pd/C	palladium on activated charcoal
PEG	polyethylene glycol
POI	protein of interest
POM	pomalidomide
PPB	plasma protein binding
PPh ₃	triphenylphosphine
PPI	protein-protein interaction
ppm	parts per million
Pr	propyl
PROTAC	proteolysis targeting chimera
PRR	pattern recognition receptor
PTM	post-translational modification
PYD	pyrin domain
P2X7	P2X purinoreceptor 7
q	quartet
quint	quintet
RHS	right-hand side of the VHL/HIF1 α interaction
RIG-I	retinoic acid inducible gene I
RING	really interesting new gene

RIPK	receptor interacting serine/threonine kinase
RLH	RIG-I-like helicase
Rpd3	reduced potassium dependency 3 protein
rt	room temperature
SAHA	suberoylanilide hydroxamic acid
s	singlet
SCF ^{β-TRCP}	Skp1-Cullin-F box complex containing Hrt1 and β -TRCP
SEC-MALS	size-exclusion chromatography coupled to multiangle light scattering
Sir2	silent information regulator 2 protein
Smac/DIABLO	second mitochondria derived activator of caspases/direct IAP binding protein with low pI
S _N Ar	nucleophilic aromatic substitution
SNIPER	specific and non-genetic IAP-dependent protein erasers
S _N 2	second-order nucleophilic substitution
SPR	surface plasmon resonance
SYK	spleen tyrosine kinase
t	triplet
<i>t</i> or <i>tert</i>	<i>tertiary</i>
TBAHS	tetrabutylammonium hydrogen sulfate
TEA	triethylamine
TEMPO	(2,2,6,6-tetramethylpiperidin-1-yl)oxyl
TFA	trifluoroacetic acid
TFEB	transcription factor EB
THF	tetrahydrofuran
TLC	thin-layer chromatography
Tle	<i>tert</i> -leucine
TLR	toll-like receptor
<i>T_m</i>	protein melting temperature
TPD	targeted protein degradation

TPSA	topological polar surface area
TP1	telomerase-associated protein
t_R	retention time
TRAF6	TNF receptor-associated factor 6
trLRR	transition LRR
Ts	tosyl group
TsCl	4-toluenesulfonyl chloride
TWIK2	two-pore domain weakly inward rectifying potassium channel 2
T2DM	type 2 diabetes mellitus
Ub	ubiquitin
UPS	ubiquitin-proteasome system
UV	ultraviolet
VBC	complex of VHL substrate recognition protein with elongins B and C
VHL	von Hippel-Lindau
WHD	winged-helix domain
XIAP	X-chromosome linked IAP
ZBG	zinc binding group
ZnF-UBD	zinc finger ubiquitin-binding domain
Zswim2	zinc finger SWIM domain-containing protein 2

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1 Introduction

1.1 Proteolysis Targeting Chimeras

Protein homeostasis is a crucial task for cells to ensure adequate cellular functions and necessitates sophisticated control mechanisms. The proteome of human cells is highly variable and depends on the spatiotemporal gene transcription and the consecutive protein translation, together with protein maintenance and degradation.¹

Dysfunctional proteins are a primary source for diseases. The traditional way to restore regular physiology is to adjust harmful protein functions with conventional inhibitors.² This approach ideally requires druglike small molecular inhibitors following the so-called rules of five by Lipinski,³ while possessing reasonable affinity to a binding site of the targeted protein. Their pharmacological effect is often ascribed to the competition with an endogenous ligand of the protein. Hence, the druggable genome in *Homo sapiens* is limited, due to a finite number of specific drug-binding sites of enzymes, receptors, ion channels and transporters.² Expanding the field of druggable proteins through the development of monoclonal antibodies⁴ and oligonucleotides⁵ already enabled novel therapeutic options.⁶

In the course of natural protein homeostasis, misfolded or no longer required proteins are degraded by intracellular components such as the ubiquitin-proteasome system (UPS). Enzymes of the enzymatic class of ligases tag considered proteins with polypeptide ubiquitin (Ub) residues as a form of post-translational modification (PTM). Under hydrolysis of adenosine triphosphate (ATP), a ubiquitin-activating enzyme (E1) activates a carboxylic acid group of ubiquitin. Subsequently, ubiquitin forms an intermediate thioester bond with an active cysteine side chain residue of the E1 ligase. Then, a ubiquitin-conjugating enzyme (E2) receives the ubiquitin residue through transthioesterification. During approximation of the ubiquitin-charged E2 ligase in complex with a ubiquitin ligase (E3) and the envisaged protein, ubiquitin is finally attached to a lysine side chain residue of the protein *via* an isopeptide bond.^{7,8} This ubiquitin transfer cascade can lead to polymeric ubiquitin side chains with homogenous or branched characteristics as well as mono- and multimono-ubiquitination of a substrate. Protein fate is regulated according to these patterns, supplying designated proteins to the 26S proteasome for proteolytic degradation.⁹

In 2001, a revolutionary strategy to manipulate cellular protein degradation was reported. With the aid of a fully synthetic molecule, a methionyl aminopeptidase was proteolytically degraded

by hijacking the UPS.¹⁰ Such synthetic molecules exhibit a tripartite structure and are termed proteolysis targeting chimeras (PROTACs). They consist of a ligand for a protein of interest (POI) and an E3 ligase binding motif, conjugated with a linker tether.¹⁰ In concert with the POI and an appropriate E3 ligase, PROTACs orchestrate a ternary complex and induce proximity of the participating proteins (Figure 1). In doing so, the E3 ligase contrives the ubiquitin transfer to the POI. Following this, the ternary complex dissociates under release of the PROTAC molecule, enabling a further catalytic cycle.¹¹ Finally, ubiquitin tagged proteins are degraded by the UPS. This protein-binding event-driven mode of action demonstrates to be superior to traditional occupancy-driven pharmacology in the case of targeting active or allosteric binding sites. Instead of structurally optimizing small molecular inhibitors in order to overcome drug resistance and diminished binding affinity to a POI, PROTAC development does not obligatorily require high affinity ligands at active sites. Hence, their basic principle is not limited to exclusive inhibition of protein function.⁸

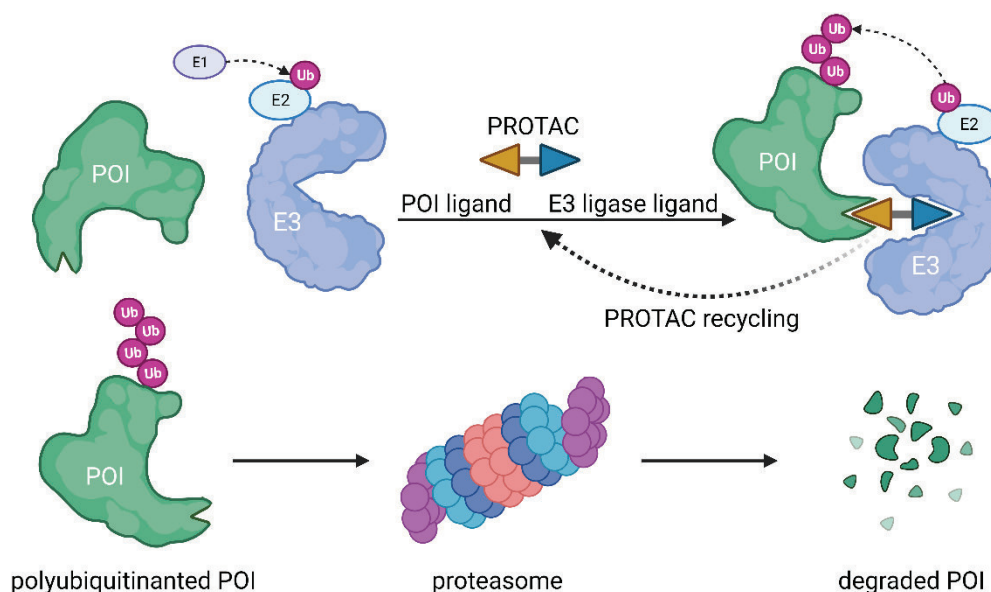


Figure 1. Degradation mechanism of a POI *via* PROTAC-mediated ternary complex formation.

The first proof-of-concept PROTACs were based on a complex phosphopeptidic ligand necessitating for microinjection to recruit the Skp1-Cullin-F box complex containing Hrt1 and β -TRCP ($\text{SCF}^{\beta\text{-TRCP}}$) E3 ligase complex.^{10,12} Improvement of the E3 ligase ligand design progressed with the identification of a cell permeable polyarginine-containing peptide ligand for the von Hippel-Lindau (VHL) E3 ligase, mimicking its endogenous substrate hypoxia-inducible factor 1 α (HIF1 α).¹³

Meanwhile smaller molecules were found to recruit various E3 ligases. In 2008, the E3 ligase mouse double minute 2 homolog (MDM2) as a key regulator of the tumor suppressor p53¹⁴ was addressed. The MDM2 imidazoline-derived ligand nutlin-3¹⁵ was implemented in an androgen receptor (AR)-targeting PROTAC (PROTAC 14, Figure 2).¹⁶ Consecutively in 2010, a successful degrader (PROTAC 4b, Figure 2) for the cellular retinoic acid-binding proteins (CRABPs) exploited the ubiquitin conjugation domain of cellular inhibitor of apoptosis protein 1 (cIAP1). This was achieved by employing a methyl bestatin derived moiety as a ligand for the involved baculoviral IAP repeat (BIR) domain.^{17,18}

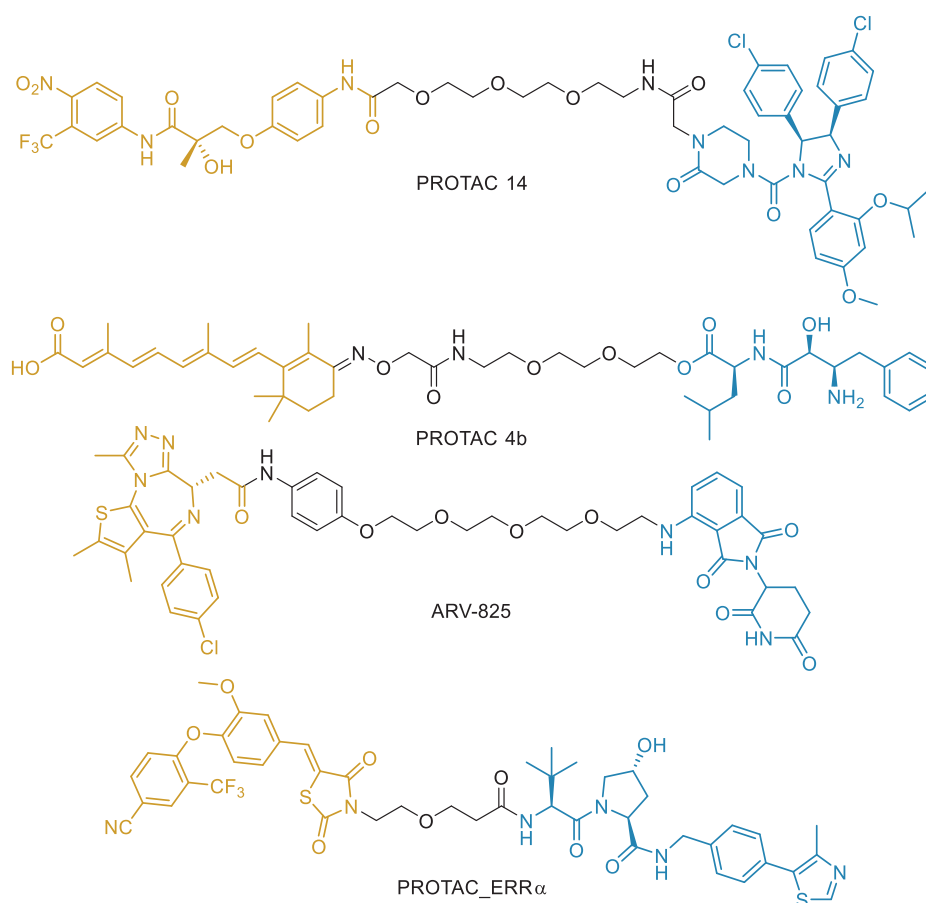


Figure 2. First reported PROTACs recruiting an E3 ligase *via* small molecular ligands. POI ligands are highlighted in ochre and E3 ligase ligands in blue.

Another milestone represented the identification of the E3 ligase cereblon (CRBN) as the primary molecular target of thalidomide, the active pharmaceutical ingredient (API) of the sedative and antiemetic drug Contergan.¹⁹ Prior to this, thalidomide gained sad notoriety as the root cause for numerous birth defects, in consequence of teratogenic properties of the *S*-configured enantiomer of the racemic drug. Contergan was ultimately withdrawn.²⁰ Nevertheless, thalidomide and its relatives pomalidomide (POM) and lenalidomide are still

applied as anticancer agents, exploiting their anti-angiogenic and anti-inflammatory effects. They are taken together as immunomodulatory imide drugs (IMiDs).²¹ First heterobifunctional degraders recruiting cereblon *via* a thalidomide²² or pomalidomide moiety (ARV-825, Figure 2)²³ were reported in 2015. Degradation of bromodomain 4 (BRD4) was accomplished with a JQ1 warhead.²⁴ After the exploration of small molecular ligands in 2012^{25,26} and 2014,²⁷ the VHL E3 ligase again attracted attention. In 2015, the first small molecular VHL ligand-based PROTACs targeted HaloTag fusion proteins,²⁸ as well as the endogenous substrates estrogen receptor (ER) alpha (PROTAC_ERR α , Figure 2), the receptor interacting serine/threonine kinase (RIPK) 2¹¹ and BRD4.²⁹

With the first crystal structure of the ternary complex consisting of the PROTAC MZ1, BRD4 and VHL, light was shed on thermodynamic aspects. Determinations of binding equilibria were performed *e.g.* by means of isothermal titration calorimetry (ITC)³⁰ or surface plasmon resonance (SPR) spectroscopy.³¹ These investigations allowed for a mathematical understanding of ternary complex formation. Calculated cooperativity (α) is understood as the ratio of dissociation constants for binary (K_D^{binary}) and ternary (K_D^{ternary}) complex formation (Figure 3). This relation considers effects from *de novo* created protein-protein interactions (PPIs) between POI and E3 ligase.

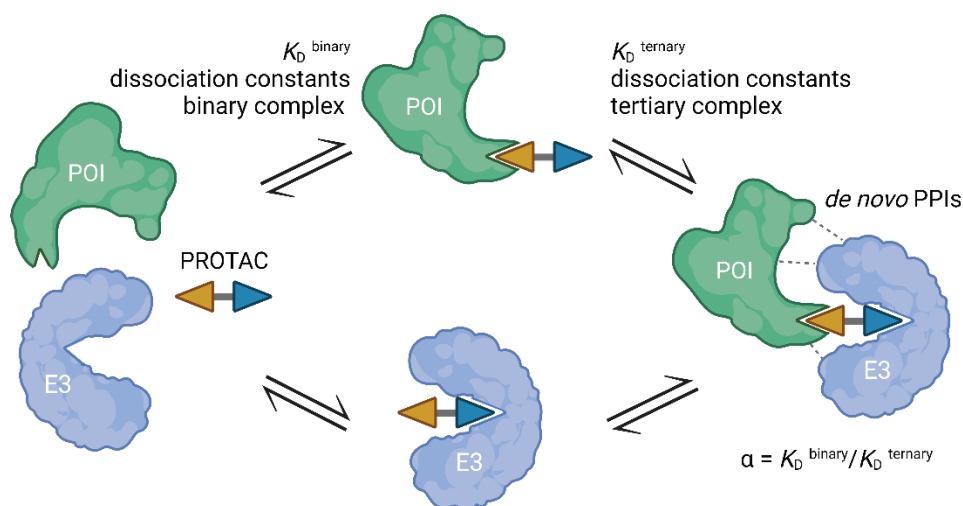


Figure 3. Determination of cooperativity (α) as an thermodynamic expression for ternary complexes. Modified from Roy *et al.*³¹

Positive cooperativity ($\alpha > 1$) describes a preferred ternary rather than binary binding behavior and presages a stable ternary complex, serving for effective POI degradation. In contrast, negative cooperativity ($\alpha < 1$) implies rather repulsive PPIs, deranging the ternary complex.^{30,31}

Nevertheless, positive cooperativity is not an obligatory prerequisite for successful PROTAC induced POI degradation.^{32,33} The extent of ternary complex formation can further be described depending on the PROTAC concentration. Since both low and high PROTAC levels may cause poor ternary complex formation, an optimal titrated PROTAC concentration aids elevated ternary complex concentrations. This bell-shaped curve illustrates the commonly referred “hook effect”. This phenomenon occurs due to the termination of ternary complex formation in consequence of excessive binary complex formation events at high PROTAC concentrations (Figure 4).^{33,34}

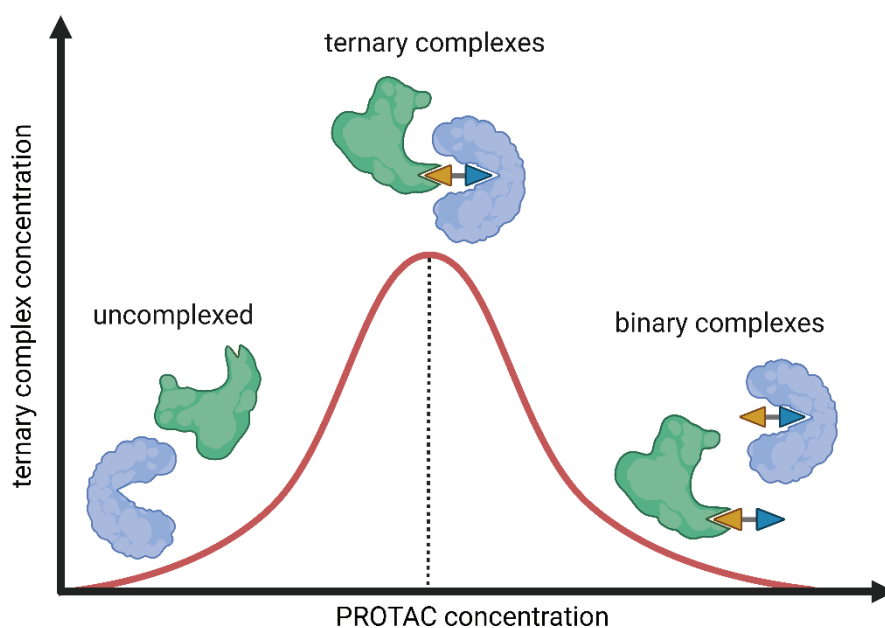


Figure 4. The “hook effect” as a limiting phenomenon owing to increasing PROTAC concentrations. Modified from Pettersson and Crews.³⁵

Once started as a proof-of-concept study, PROTACs have emerged to a new chance in facing diseases and malignancies. Numerous heterobifunctional degraders have been developed for the depletion of multiple POIs, from which at least 15 candidates entered clinical trials by now, with potential anticancer agents going ahead. The most advanced PROTACs ARV-110 and ARV-471 (structures not shown) aim for the degradation of oncogenic drivers, *i.e.* the AR or the ER, respectively. Both represent well-established target structures in either prostate or breast cancer.⁷

1.2 Exploration of NLRP3 as a Promising Drug Target

Jawed vertebrates are equipped with a multifaceted immune system to defend against pathogen invasion of various origin and to detect tissue damage and metabolic derangements. It can be divided into an innate and an adaptive system, while each is armed with cellular and humoral components.^{36,37} Upon contact to pathogen-associated molecular patterns (PAMPs),³⁸ such as lipopolysaccharide (LPS) from the outer plasma membrane of Gram-negative bacteria,³⁹ or out-of-context endogenous damage-associated molecular patterns (DAMPs)⁴⁰ like ATP,⁴¹ immune cells of the innate immune system react in an unspecific mode of action. Such molecular structures can be identified by germline-encoded pattern recognition receptors (PRRs).³⁸ These are localized on the cell membrane or in the cytoplasm of neutrophils, monocytes, macrophages, dendritic cells and natural killer cells.^{36,42} Toll-like receptors (TLRs) are membrane-bound PRRs.⁴³ C-type lectin receptors (CLRs) are mainly resided in membranes, while some are soluble proteins.⁴⁴ Members of the nucleotide-binding oligomerization domain (NOD)-like receptor (NLR) family^{45,46} and the retinoic acid inducible gene I (RIG-I)-like helicases (RLHs) are cytosolic sensor proteins.^{47,48} These reported types of PRRs are able to initiate diverse signal cascades in a synergistic manner causing a rapid immune response.

One representative of the NLR family is the nucleotide-binding oligomerization domain, leucine-rich repeat and pyrin domain-containing protein 3 (NLRP3), also referred to as cryopyrin⁴⁹ or NALP3.⁵⁰ It is encoded by the eponymous gene *NLRP3*, also called *NALP3* or *CIAS1*.⁵⁰ The tripartite protein NLRP3 consists of an N-terminal pyrin domain (PYD), a central NACHT domain and a C-terminal leucine-rich repeat (LRR) domain.⁴⁹ The name of the triple-ATPase domain NACHT is derived from proteins itself containing the domain, *i.e.* the neuronal apoptosis inhibitory protein (NAIP), the MHC class II transcription activator (CIITA), the incompatibility locus protein from *Podospora anserina* (HET-E) and the telomerase-associated protein (TP1).⁵¹ However, the NACHT domain is subdivided into a nucleotide-binding domain (NBD), the helical domain 1 (HD1), the winged-helix domain (WHD) and the helical domain 2 (HD2), Figure 5).⁵²

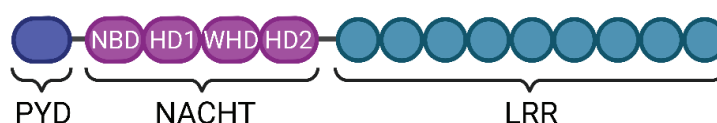


Figure 5. Schematic structure of NLRP3 monomer.

The sequence of canonical NLRP3 inflammasome formation can be dissected into a consecutive dual step process (Figure 6). During the priming step, PRRs or cytokine receptors are stimulated, in turn activating the transcription factor nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NF- κ B). In case of TLR- and the interleukin-1 receptor (IL-1R)- signaling, NF- κ B is activated *via* the myeloid differentiation primary response 88 (MyDD88), interleukin-1 receptor-associated kinases 1 and 4 (IRAK1/4) and TNF receptor-associated factor 6 (TRAF6) downstream cascade. As a consequence, the expression of the inactive interleukin (IL) proform pro-IL-1 β as well as the expression of NLRP3 are upregulated.^{53–55} Beside transcriptional priming, also non-transcriptional priming⁵⁶ through PTMs of NLRP3, such as (de-)ubiquitination,⁵⁷ (de-)phosphorylation⁵⁸ and (de-)sumoylation⁵⁹ have been reported. These modifications regulate aspects of protein function in a more rapid manner than by transcriptional priming.^{60,61}

The second step constitutes the actual activation mechanism of the NLRP3 inflammasome. It is generally ascribed to potassium efflux,⁶² lysosomal disruption, and mitochondrial dysfunction. Potassium efflux is facilitated, *e.g.* by bacterial ionophores, including nigericin⁶³ and gramicidin,⁶⁴ the ATP-dependent opening of the ion channel P2X purinoreceptor 7 (P2X7),⁶⁵ or by the two-pore domain weakly inward rectifying potassium channel 2 (TWIK2). TWIK2 again is modulated by P2X7-mediated sodium and calcium influx.⁶⁶ In order to clear accumulated phagocytized particulates such as endogenous monosodium urate (MSU) crystals,⁶⁷ amyloid- β aggregates⁶⁸ or even asbestos⁶⁹ as an exogenous contaminant, lysosomes lose their membrane integrity. This is caused by harsh digestion conditions, enabling the release of cathepsins, thus increasing extracellular ATP levels and finally supporting potassium efflux.^{70,71} In addition, mitochondrial dysfunction is hypothesized to drive NLRP3 inflammasome activation by the release of potential immunostimulatory oxidized mitochondrial DNA (ox-mtDNA) and mitochondrial reactive oxygen species (mtROS) into the cytosol.^{72–74}

Once the NLRP3 sensor is stimulated, the serine/threonine NIMA-related kinase 7 (NEK7) interacts with the LRR and parts of the NACHT domain of NLRP3.⁷⁵ Adenosine diphosphate (ADP) in the NBD of the NACHT domain is exchanged for ATP,⁷⁶ allowing NLRP3 to leave the autoinhibited conformation. The adaptor protein apoptosis-associated speck-like protein containing a caspase recruitment domain (CARD) (ASC) is recruited *via* PYD-PYD interaction, aggregating ASC specks in the shape of prion-like fibrils.⁷⁷ ASC then binds the zymogen pro-caspase-1 by CARD-CARD interaction,^{60,78} orchestrating the NLRP3 inflammasome as a supramolecular complex. Autoproteolysis of aggregated pro-caspase-1 gives active caspase-1.

The effector protease processes pro-IL-1 β and pro-IL-18 into their corresponding proinflammatory cytokines IL-1 β and IL-18. Caspase-1 also mediates the cleavage of gasdermin D (GSDMD) into its pore-forming N-terminal fragment gasdermin N (GSDMD-N).^{79,80} The matured proinflammatory cytokines IL-1 β and IL-18 subsequently can cross the cell membrane and swelling eventually leads to the rupture of the cell. This lytic form of programmed cell death is referred to as pyroptosis.^{81–83}

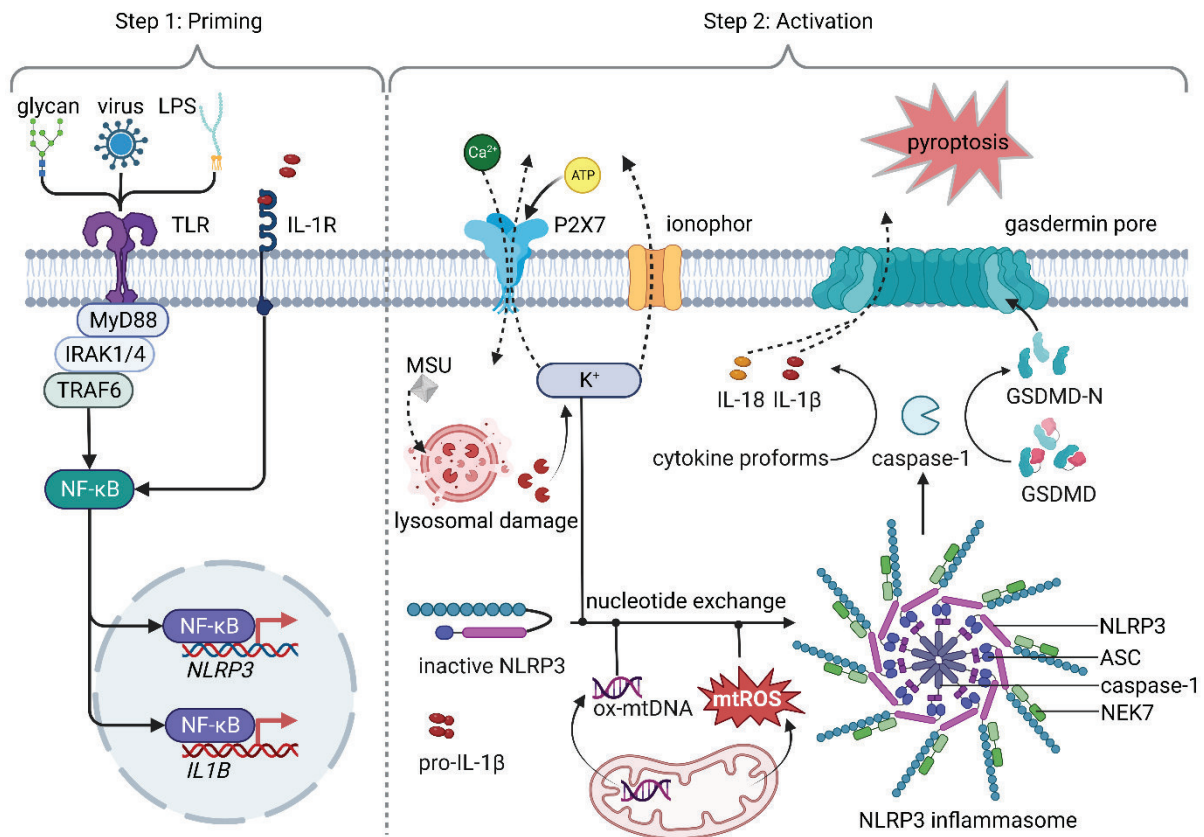


Figure 6. Canonical priming and activation of the NLRP3 inflammasome. Modified from Van Den Eeckhout *et al.*⁵⁴

In addition to the canonical activation of the NLRP3 inflammasome, cytoplasmic sensing of LPS by human caspases 4 and 5 leads to a non-canonical NLRP3 activation.^{84,85} A further so-called alternative pathway activates NLRP3 *via* human caspase-8 and depends on LPS-mediated stimulation of TLR4, thus mediating RIPK1 downstream signaling.⁸⁶ Beside the intensive research efforts towards the NLRP3 inflammasome, also others such as the NLR family CARD domain-containing protein 4 (NLRC4) inflammasome^{87,88} and the absent in melanoma 2 (AIM2) inflammasome^{89,90} attracted increasing attention. They serve as upstream sensors for further potential pathogens, thus activating downstream caspase-1.

NLRP3 might be assumed as an antagonistically pleiotropic gene, since the function of its transcribed protein NLRP3 is to unleash a powerful immune response against diverse pathogen invasions. However, abnormal inflammation processes are observed in patients exhibiting gain-of-function mutations in the *NLRP3* gene. The accumulation of sterile inflammation triggers especially makes patients of advanced age vulnerable to inflammasome-related diseases.^{91,92} Autosomal dominant mutations in the *NLRP3* gene provoke the rare cryopyrin-associated periodic syndrome (CAPS), which includes the three diseases familial cold autoinflammatory syndrome (FCAS), Muckle-Wells syndrome (MWS) and the neonatal-onset multisystem inflammatory disorder (NOMID). Periodic fever, blood neutrophilia and specific skin inflammation are common hallmarks. Aberrant NLRP3 inflammasome formation also plays a major role in disorders of the central nervous system or autoinflammatory diseases such as rheumatoid arthritis and inflammatory bowel disease (IBD). NLRP3-associated metabolic afflictions especially comprise type 2 diabetes mellitus (T2DM), when it comes to low-grade chronic inflammation as a reason for insulin resistance, and liver steatosis ending up in a nonalcoholic steatohepatitis (NASH).⁹²

Therefore, facing excessive immune reactions by targeting the NLRP3 inflammasome gained tremendous momentum in recent drug development efforts. Since NLRP3 is highly diversely triggered and operates in inflammasome protein complexes, multiple therapeutic interventions are conceivable. Those might be the blockade of upstream events in the priming and activation mechanisms,⁹³ alteration of proteins' PTMs or the manipulation of ASC and caspase-1 functions.⁹² Cytokines being linked to NLRP3 are rendered harmless either by the recombinant IL-1 receptor antagonist anakinra, the IL-1 β and IL-1 α affine soluble decoy receptor rilonacept or the human IL-1 β monoclonal antibody canakinumab.⁹⁴ However, controlling inappropriate inflammation reactions straight *via* the IL-1 β axis, inhibits the function of other pathways. This may raise the danger of unintended immunosuppression. Beside chronic IL-1 inhibition and neutropenia, further limitations come along with the required parenteral administration of those biologicals. Arising local side effects and the periodic application argue against such agents.^{95,96} Hence, small molecules that are directly interfering with NLRP3 while allowing for oral application are appreciated.

Identification of potential pharmacophores tackling non-established drug targets remains a substantial challenge. A phenotypical screening approach laid the foundation for the inhibitor design, concerning NLRP3-mediated IL-1 β release from LPS-activated human monocytes and macrophages. Treatment with the sulfonylurea antidiabetic drug glyburide unexpectedly decreased IL-1 β release from human monocytes with a half-maximal inhibitory concentration

(IC₅₀) of 12 μ M. However, its analog glipizide, did not inhibit cytokine release, hypothesizing the mechanism to be independent from inhibition of their molecular target, responsible for the antidiabetic effect. For this reason, two structural derivatives, *i.e.* CP-424,174 and CP-412,245 (structures not shown), both including a diaryl sulfonylurea moiety, were tested. They were found to sufficiently block IL-1 β liberation with IC₅₀ values of 0.21 μ M and 0.26 μ M, designating them as cytokine release inhibitory drugs (CRIDs).⁹⁷ A close relative of the first reported CRIDs is CRID3, also known as CP-456,773. It is the most prominent NLRP3 inflammasome inhibitor to date and was first presumed to interact with glutathione *S*-transferase Omega 1-1.⁹⁸ Later, CRID3 was redefined as MCC950 (Figure 7) and was linked to the NLRP3 inflammasome for the first time. An IC₅₀ of 7.5 nM for mice bone marrow-derived macrophages (BMDMs) and a value of 8.1 nM for human monocyte-derived macrophages (HMDMs), regarding IL-1 β release were exhibited.⁹⁹ Target deconvolution was achieved using a drug affinity responsive target stability (DARTS) approach combined with a SPR spectroscopy strategy.¹⁰⁰ It was discovered, that MCC950 directly binds to the NACHT domain of NLRP3 in a reversible manner. Hence, suppressing NACHT's ATPase activity and strengthening the autoinhibited, so-called 'closed' NLRP3 conformation.^{100,101} By now, the crystal structure of MCC950 bound to the central NACHT domain of full-length human NLRP3 in the inactive conformation was solved by means of cryogenic electron microscopy (cryo-EM).⁵² Intertwined LRR domains of two NLRP3 proteins stabilize a corresponding homodimer, which in turn conglomerates as a pentamer of homodimers. Finally, the NLRP3 inflammasome assembles as a decamer. The sulfonylurea moiety of MCC950 is stuck between the arginine residues of R351 from the NBD, R578 from the HD2 and the alanine residue of A228 from the NBD. The characteristic hexahydro-*s*-indacene on the western part of the molecule forms multiple interactions with M408 and V414 of the HD1, with T439, Y443 and T524 of the WHD and with F575 of the HD2. Furthermore, it interacts with M661 of the transition LRR (trLRR), which spans between NACHT and LRR domain. While the furan of MCC950 is adjacent to proline 352 from the NBD, its terminal tertiary alcohol interacts with Y632 and L628 from the HD2. Hence, the tertiary alcohol protrudes out of the binding pocket.⁵²

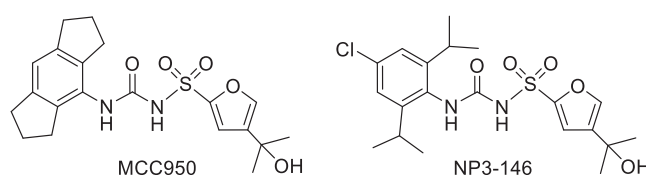


Figure 7. MCC950 and NP3-146¹⁰² as ligands of the central NACHT domain of NLRP3.

Although exerting beneficial effects in animal models facing a broad range of inflammatory diseases, MCC950 was withdrawn during the drug approval process, due to the liver toxicity issues in the phase II clinical trial.⁹² Notwithstanding, MCC950 is the best investigated and highly specific pharmacological tool compound for judicious NLRP3 inflammasome exploration.⁹² Recent studies introduce the development of structural derivatives of MCC950. These are listed as potential NLRP3 inhibitors^{103–105} or are instrumentalized as pharmacological tool compounds in terms of fluorescent,¹⁰² radio-labeled¹⁰⁶ or cross-linking photoaffinity probes (Figure 8).^{100,101,107,108}

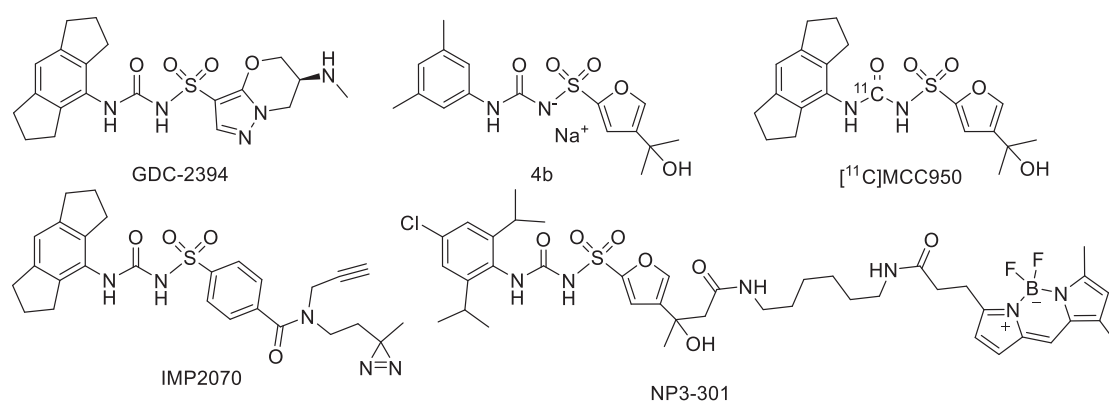


Figure 8. NLRP3 ligands containing the characteristic diaryl sulfonylurea moiety of MCC950.

Apart from MCC950, additional NLRP3 inhibitors ranging from synthetic small molecules to natural products have been reported (Figure 9). CY-09 was identified as a reversible NLRP3 inflammasome inhibitor in the course of a compound library screening of inhibitors for the cystic fibrosis transmembrane conductance regulator. Pulldown experiments with a biotinylated CY-09 derivative revealed the NACHT domain of NLRP3 as the target structure, interfering with the ATP-binding motif. This was further confirmed by molecular docking. Upon nigericin or MSU stimulation, inhibition of IL-1 β release from LPS primed murine BMDMs was achieved at micromolar concentrations.¹⁰⁹

The anti-allergic drug tranilast (Figure 9), suppresses the immunoglobulin E (IgE)-mediated release of histamine from mast cells. Hence, it is used to treat inflammatory diseases, *e.g.* bronchial asthma, allergic conjunctivitis and atypical dermatitis. Later on, tranilast was proven to disturb the NLRP3 inflammasome assembly. The central NACHT domain was pulled down with the aid of a biotin probe. However, NACHT's ATPase activity was not influenced by tranilast. Compared to MCC950, tranilast had a 400 times less potent activity, regarding inhibition of MSU-induced IL-1 β release from BMDMs.^{110–112}

3,4-Methylenedioxy- β -nitrostyrene (MNS, Figure 9) is an inhibitor of the spleen tyrosine kinase (SYK). In addition, MNS blocked IL-1 β secretion from murine BMDMs upon ATP stimulation with an IC₅₀ value of 2 μ M. In experiments with SYK-knockout murine BMDMs, MNS still abolished IL-1 β release, indicating a SYK kinase independent NLRP3 inhibition. A biotinylated analog of MNS sufficiently pulled down the NOD and LRR domains of NLRP3. The probe was competed off by excess addition of the designated NLRP3 inhibitor BAY 11-7082 (Figure 9). MNS-dependent inhibition of the ATPase activity of the NACHT domain was confirmed by measurement of free phosphate. These findings substantiate NLRP3 to be an additional molecular target of MNS.¹¹³ The applied phenyl vinyl sulfone derivative BAY 11-7082 was intended as an inhibitor of the NF- κ B pathway. Later, it was found to inhibit the ATPase activity of NLRP3, independent from NF- κ B. This finding was ascribed to alkylation of essential nucleophilic residues of the protein sidechains *via* Michael addition, thus impeding inflammasome activation in an irreversible covalent fashion.¹¹⁴

Dapansutrine is an advanced NLRP3 inhibitor and also known as OLT1177 (Figure 9). The β -sulfonyl nitrile is currently evaluated in clinical trials for a diverse field of indications, *e.g.* osteoarthritis, acute gout flare and heart failure.^{115,116} The inhibition of IL-1 β release from macrophages of mice cell line J774A.1 upon dapansutrine treatment was comparable to MCC950 intervention. Dapansutrine's activity is attributed to the inhibition of the ATPase activity of NLRP3.¹¹⁷

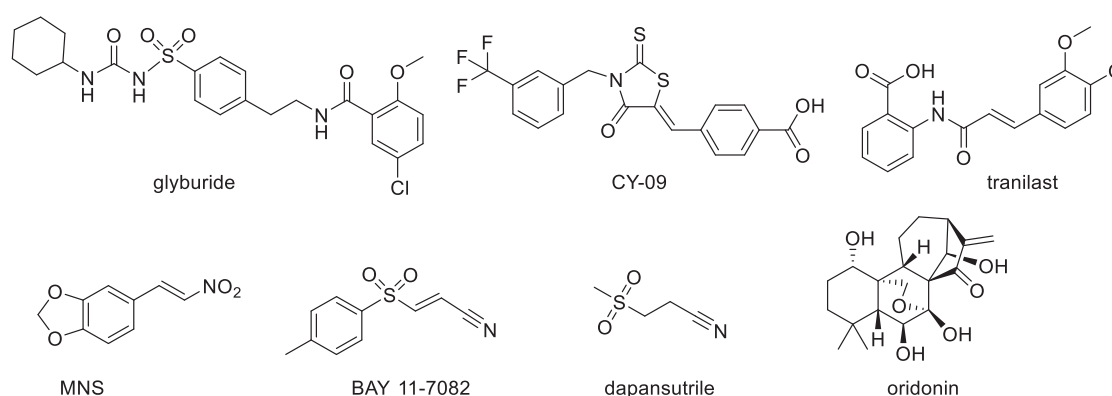


Figure 9. Collection of reported NLRP3 inhibitors.

Beside synthetic inhibitors, also natural substance scaffolds were identified as NLRP3 inhibitors.¹¹⁸ Oridonin is an *ent*-kaurane diterpene of the herb *Rabdosia rubescens*. Preparations of the herb are used in traditional Chinese medicine for a large field of indications, but in particular for treating inflammatory disorders.^{119,120} Healing effects are especially ascribed to the major active ingredient oridonin (Figure 9). It covalently binds to C279 in the NACHT

domain *via* the α,β -unsaturated carbonyl unit acting as a Michael acceptor. As a consequence, the interaction between NLRP3 and NEK7, which is a licensing step in inflammasome activation,^{52,75} is perturbed. A synthesized hydrogenated oridonin derivative, lacking the carbon-carbon double bond, was unable to bind the thiol residue of C279.^{119,120}

1.3 Therapeutic Potential of HDAC6 as a Unique HDAC Family Member

The genetic information of cells is encoded in the form of a defined sequence of deoxyribonucleic acid (DNA). For compact packaging, DNA is wrapped with a length of 145 to 147 base pairs around an octameric histone protein complex, forming nucleosomes (Figure 10). These represent the essential building blocks of chromatin.^{121,122} The octameric configuration of the core particles is based on four histone species, which are H2A, H2B, H3 and H4. Histones H3 and H4 constitute a tetramer and are accompanied by two H2A/H2B dimers.^{123,124} The core histones are characterized by the abundance of basic amino acids. Their positively charged residues enable strong interactions with the negatively charged phosphate residues of the DNA, acting as a coil. Especially the N-terminal tails of histones are frequently modified, as they protrude out of these globular structured proteins and thus are predestined for PTMs.¹²⁵ Almost 60 years ago, it was found that minor changes in the histone structure, such as acetylation or methylation,¹²⁶ influence DNA function.^{125,127} These alterations in histone and chromatin structure exclude DNA sequence changes. They are centralized in the concept of epigenetics¹²⁸ and trace back to year 1942,¹²⁹ prior to the elucidation of the genetic code in 1961.¹³⁰ Various histone derivatizations beyond acetylation and methylation have been reported in the course of time, *e.g.* phosphorylation, ubiquitylation, sumoylation, ADP ribosylation, deamination and proline isomerization.^{123,131} Acetylation and opposite deacetylation gained particular attraction, as they were the first modifications being linked to gene activity.^{125,127} Acetylation of histone ϵ -amino lysine residues leads to abrogation of DNA interactions with the positively charged amino acids. In turn, the chromatin structure unfolds and the DNA is exposed for replication or transcription.^{132,133} This less compact, decondensed form of chromatin is named euchromatin, while the condensed rather inactive form of chromatin, *i.e.* heterochromatin, is characterized by hypoacetylated histones.¹²⁴ The balance between acetylation and deacetylation depends on the existence of histone acetyltransferases (HATs)^{134–136} and histone deacetylases (HDACs).^{137,138}

HATs transfer acetyl residues by using the cofactor acetyl-coenzyme A. They can be divided into type-A and type-B, from which members of the latter type acetylate free histones within the cytoplasm. Type-A HATs, being present in the nucleus, are further subdivided into three

families according to their sequence homology and conformational structure, *i.e.* CBP/p300, GNAT and MYST.^{139,140} However, HDACs support the remodeling and stabilization of chromatin by deacetylating ϵ -N-acetyl lysine residues, in turn repressing transcription. The enzyme class of human HDACs comprises 18 members, from which eleven are zinc-dependent and seven need oxidized nicotinamide adenine dinucleotide (NAD⁺) as cofactor. In accordance with their analog enzymes from yeast, HDACs are sorted into four classes (I-IV). Differences among them relate to their tissue distribution, subcellular localization, substrate specificity and binding partners. The homologs of yeast reduced potassium dependency 3 protein (Rpd3) are represented in Class I, including HDACs 1, 2, 3 and 8. They are ubiquitously expressed and localized in the nucleus. HDACs 4, 5, 6, 7, 9 and 10 are class II members and share similarities with histone deacetylase A1 (Hda1) from yeast. HDACs 4, 5, 7 and 9, which belong to class IIa, occur in the cytoplasm and the nucleus, whereas class IIb HDACs 6 and 10 are cytoplasmic enzymes. Sirtuins 1 to 7 represent the NAD⁺-dependent class III HDACs and are similar to the silent information regulator 2 protein (Sir2) from yeast. HDAC class IV includes HDAC11, which has just little sequence conformity to HDACs from class I and II.^{141,142}

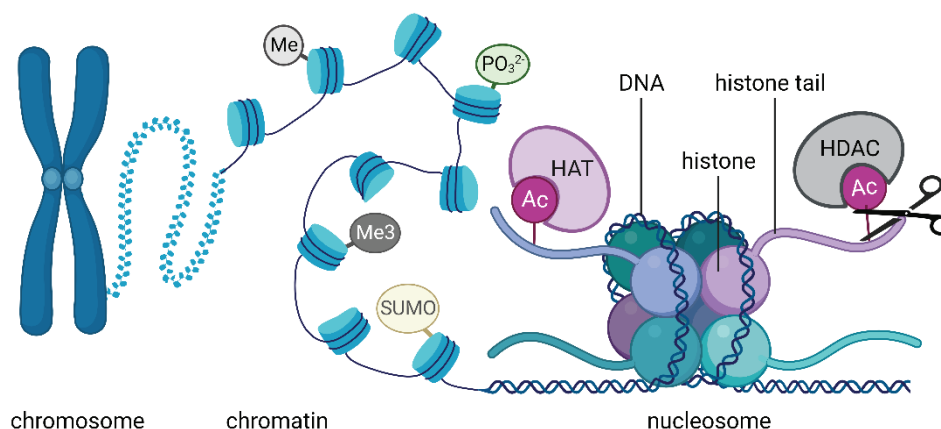


Figure 10. A disentangled chromosome illustrating nucleosomes as pivotal building blocks and substrate for PTMs.

Due to the powerful function of HDACs as gene silencers and their ability to deacetylate a large variety of non-histone proteins, investigations revealed a deregulated expression of several HDAC isoforms in several primary human cancer tissues.^{143–147} The repression of genes encoding for tumor suppressors,¹⁴⁸ differentiation factors,¹⁴⁹ cell cycle inhibitors,¹⁵⁰ or apoptosis inducers¹⁵¹ fuels tumorigenesis and can be opposed by HDAC inhibitors.^{142,152} Abrogation of aberrant HDAC activity can trigger complex mechanisms and depends on the cellular context. Since unselective HDAC inhibitors arbitrarily target various HDACs and their corresponding substrates, they may cause inadvertent effects.¹⁵³

An appropriate pharmacophore for inhibitors of zinc-dependent HDACs includes a hydrophobic capping group and a hydrophobic linker-connected zinc binding group (ZBG). An appropriate ZBG is crucial for interactions with a zinc cation in the catalytic center, which is highly conserved throughout the HDAC enzyme class.^{154,155} Structural adaptations to this pharmacophore, enhancing selectivity for individual isoforms, have been made, such as the use of an *ortho*-aminoanilide, instead of hydroxamic acid, as ZBG in HDAC I and II inhibitors.^{156,157} An even more advanced approach towards improved selectivity is the introduction of a so-called “foot-pocket” unit to occupy an internal cavity.^{158,159} To date, four HDAC inhibitors are approved by the Food and Drug Administration of the United States of America (FDA). These are the hydroxamates vorinostat (suberoylanilide hydroxamic acid, SAHA), panobinostat and belinostat, as well as the depsipeptide romidepsin (Figure 11). The latter constitutes a prodrug, which upon reduction of the disulfide reveals a thiol ZBG. Another HDAC inhibitor is the *ortho*-aminoanilide tucidinostat, which is approved by the Chinese National Medical Products Administration (NMPA).¹⁴²

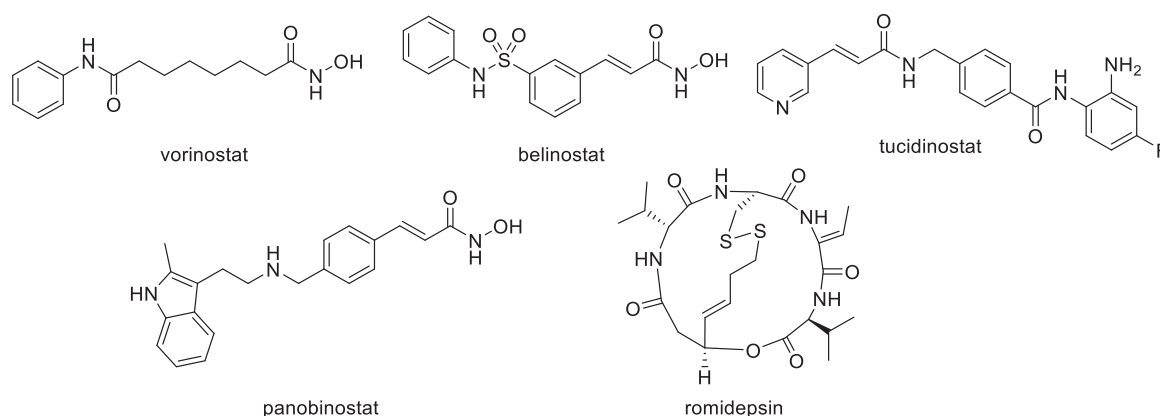


Figure 11. Structure of approved HDAC inhibitors.

Of eminent interest is the selective inhibition of class IIb member HDAC6, because of its atypical structural and functional features within the enzyme family. HDAC6 contains two catalytic domains (CDs), *i.e.* CD1 and CD2,^{160,161} a dynein binding domain and a unique zinc finger ubiquitin-binding domain (ZnF-UBD).^{162,163} It is recognized that *in vivo*, HDAC6 tends to deacetylate various non-histone proteins, such as α -tubulin,¹⁶⁴ heat shock protein 90 (HSP90),¹⁶⁵ and cortactin,¹⁶⁶ instead of histones.¹⁶⁰ In doing so, a plethora of proteins is influenced. Increased chemotactic cell motility was attributed to α -tubulin deacetylation upon elevated HDAC6 levels.¹⁶⁴ Deacetylation of HSP90 supports its chaperone function. Hence, proper folding of oncogenic kinases such as the BCR-ABL kinase, encoded by the *BCR-ABL* (breakpoint cluster region gene/Abelson proto-oncogene) fusion gene, is maintained.¹⁶⁷

Apart from deacetylase activity, an inimitable competence of HDAC6 is to bridge polyubiquitinated proteins and dynein motor proteins. This is accomplished by its ZnF-UBD dynein binding domain. Hence, misfolded and ubiquitin-tagged proteins are transported along microtubules to the microtubule-organizing center (MTOC). Subsequently, the supplied proteins conglomerate as aggresomes.^{163,168} These are eventually subjected to a proteasome-independent clearance system. Physiologically, this cellular protection mechanism avoids cytotoxicity by maintaining the sequestration of dysfunctional proteins *via* autophagy, even if the UPS is incapable.^{169–172} The mentioned mechanisms, among other interactions, make HDAC6 an interesting drug target for a large indication area. Inhibition of HDAC6, often in combination with established drugs, has shown beneficial effects in oncology,¹⁷³ autoimmunity¹⁷⁴ and neurology.¹⁷⁵

In order to achieve selective HDAC6 inhibition, the already outlined pharmacophore of HDAC inhibitors can be further functionalized. By introduction of a more bulky and intricate capping group¹⁷⁶ and by substitution of alkyl linkers for benzyl linkers, the selectivity profile is optimized. These structural features are in place in the highly selective HDAC6 inhibitors tubastatin A,¹⁷⁷ nexturastat A¹⁷⁸ and their relatives.^{179,180} Ricolinostat is characterized by its voluminous capping group and is investigated in advanced clinical studies as an HDAC6-preferential inhibitor (Figure 12).¹⁴² An exchange of hydroxamic acid as ZBG, *e.g.* for a mercaptoacetamide or trifluoroacetylthiophene moiety was reported, albeit hydroxamates were not outperformed in terms of selectivity.^{181,182}

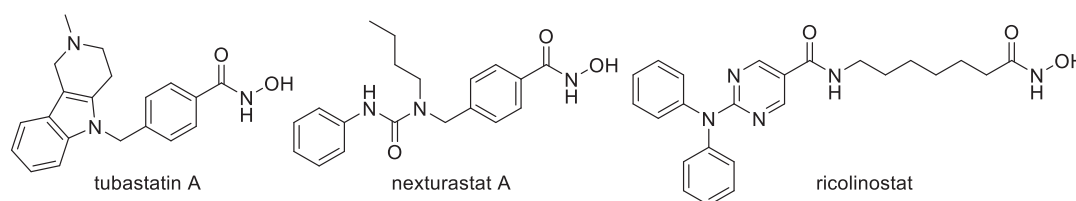


Figure 12. Selective HDAC6 inhibitors.

Due to the versatile character of serious diseases in the field of cancer or neurogenerative disorders, alternative drug modality approaches beside plain inhibition are appreciated. Merging of pharmacophores for HDAC6 and additional targets led to the development of dual inhibitors.^{183–186} Moreover, the development of PROTACs targeting HDACs, in particular HDAC6, has been pursued in the past years.^{187–190}

1.4 The Relation of NLRP3, HDAC6 and Autophagy

A growing body of research focuses on the mechanisms underlying the role of HDAC6 in the NLRP3 inflammasome pathway.¹⁷² HDAC6 is known to post-translationally modify or directly interact with a variety of autophagy-associated players *via* both its catalytic and binding domains. Examples are the lysosome master controller transcription factor EB (TFEB),^{191–193} the transcription factor forkhead box protein O1 (FOXO1)¹⁹⁴ or the dynein adaptor proteins.¹⁶⁴ Especially the interplay between the scaffolding protein p62 and HDAC6 is crucial for autophagy processes.¹⁹⁵ This may affect the formation of the NLRP3 inflammasome by depletion of involved components or inflammation triggers owing to autophagy.¹⁹⁶ Another study reports on HDAC6 bridging dynein motor proteins and ubiquitinated NLRP3. Hence, the inflammasome sensor is forwarded from the trans-Golgi network along the microtubules to the MTOC in an aggresome-like fashion. Microscopy experiments suggest a colocalization of NLRP3, ASC and procaspase adjacent to the MTOC. This finding ascribes HDAC6 to a supportive role in NLRP3 inflammasome formation in mouse cells.¹⁹⁷ Intriguingly, it was found that HDAC6 is dispensable for NLRP3 transport to the MTOC in human cells.¹⁹⁸ Elevated levels of acetylated α -tubulin, facilitated by inhibition of the catalytic domain of HDAC6 with tubastatin A, ricolinostat or tubacin, were expected to promote inflammasome activation in accordance with an earlier report on deacetylase sirtuin 2.¹⁹⁹ However, the opposite was true for HDAC6, since an inhibition of the NLRP3 inflammasome in terms of diminished IL-1 β secretion was observed.¹⁹⁷ Nevertheless, the suggestion of HDAC6-dependent inhibition of the NLRP3 inflammasome was underpinned by both HDAC6 inhibitors²⁰⁰ and PROTACs.^{198,201} In each study, NLRP3-dependent IL-1 β release was abolished under treatment with the respective compounds. It is recognized that HDAC6 inhibition intrudes the NLRP3 pathway by interfering with TLR-signaling.¹⁹⁸ Other reports on the contrary provide evidence for an HDAC6-dependent inhibitory effect on inflammasome formation, supported by HDAC6 knockout experiments.²⁰² However, HDAC6 and in particular the interplay with autophagy adds another layer of complexity to the understanding of NLRP3 inflammasome formation. The distinct mechanisms remain enigmatic and need to be clarified.¹⁹⁶

1.5 Objectives of the Dissertation

The ambition of this work is to pave the way for new drug modalities in targeting the NLRP3 inflammasome. To the best of my knowledge, there are no dedicated NLRP3 PROTACs published in a peer-reviewed journal at this juncture. In order to obtain tripartite heterobifunctional degraders, an existing NLRP3 inhibitor scaffold will be adapted. The POI ligands have to be equipped with a reasonable exit vector for consecutive linker attachment.

Prior to this, a set of sulfonylureas will be conceptualized. Since diaryl sulfonylureas are known for chemical instabilities in different media, the stability of synthesized compounds will be investigated by high-performance liquid chromatography (HPLC). By taking this knowledge into account, different NLRP3 ligands, comprising variable exit vector strategies, will be realized.

In order to evaluate cell permeability of large NLRP3 ligands as a prerequisite for the envisaged PROTAC design, both, fluorescent and biotinylated probes, will be prepared. In the next step, they will be subjected to inflammasome inhibition assays, and their cellular behavior will be monitored by means of confocal microscopy.

The PROTAC-dependent recruitment of intended E3 ligases demands the use of adequate ligands, whose syntheses will be conducted. Installation of different linker tethers between a POI and an E3 ligase ligand is challenging. Attention has to be paid to compatibility issues, regarding derivatization of functional groups and the cleavage of protecting groups. Therefore, the design of bifunctionalized linker portions plays a major role.

Finally, two degrader sets will be designed and synthesized for examining NLRP3 as an appropriate substrate for targeted protein degradation (TPD). For this purpose, the obtained PROTACs will be subjected to a thermal stability assay, as an indicator for protein binding. Simultaneously, selected PROTACs will be chromatographically investigated towards first physicochemical and pharmacokinetic properties. Afterwards, protein levels of NLRP3 in selected cell lines upon PROTAC treatment can be determined, as an evidence for ternary complex formation and degradation.

Another project aims at the development of degraders targeting the epigenetic key player HDAC6. Although being faced by a PROTAC approach for several times, hydroxamate-based compounds are detrimental for multiple reasons. As already outlined, alternative ZBGs are appreciated in the design of both HDAC6 inhibitors and PROTACs. Hence, a series of selective HDAC6 degraders employing a non-hydroxamate ZBG motif will be realized.

2 Development of the First Non-Hydroxamate Selective HDAC6 Degraders

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Please refer to Appendix I for the publication's full text and supporting information.

2.1 Publication Summary

Containment of aberrant HDAC function, by means of active site inhibition or TPD approaches, constitutes a promising strategy for the pharmacological treatment of cancer or diseases related to non-histone proteins. Especially isoform-specific inhibition of HDAC6 is aimed to decrease adverse effects, being observed for unselective HDAC inhibition.^{142,153} Due to its unique structure and the accompanying atypical functions, plain inhibition of a single catalytic domain of HDAC6 may be inferior in comparison to reversible chemical knockout *via* induced proteolytic degradation. All hitherto reported potent selective HDAC6 PROTACs are based on a hydroxamic acid moiety as ZBG,^{187–189,203,204} targeting the active site of CD2.^{142,161} However, this class of compounds is prone to form reactive isocyanate electrophiles as a consequence of Lossen rearrangements, thus causing mutagenic and genotoxic effects.²⁰⁵ The exchange of hydroxamic acid as ZBG is therefore highly appreciated and was already implemented in inhibitors by the installation of mercaptoacetamides or thiols.²⁰⁶ Recently published patent literature drew attention to a pyrimidine-linked difluoromethyl-1,3,4-oxadiazole (DFMO; not to be confused with difluoromethylornithine) warhead as a potential HDAC6 selective ligand scaffold.²⁰⁷ Hence, it served as a starting point for the design of the non-hydroxamate HDAC6 PROTACs in this project.

Two POI ligands were synthesized, differing in their exit vector orientation, with a protected aminomethyl moiety either occupying the *meta*- or *para*-position of a terminal phenyl ring. Docking studies of their corresponding hypothetical acetylated analogs underlined the *meta*-substituted ligand to be rather solvent exposed, while the acetyl group of the *para*-substituted derivative tended to interact with amino acid residues of the cavity. Nevertheless, heterobifunctional degraders based on both POI ligands were developed to address the most

well-established E3 ligases in the PROTAC field, *i.e.* CRBN and VHL. For this purpose, the high affinity ligands 4-fluoro-thalidomide²⁰⁸ and a *tert*-butyloxycarbonyl (Boc)-protected VHL ligand were synthesized.^{209,210} To consider influences of linker and in particular degrader polarity, both, polyethylene glycol (PEG) and alkyl linkers, were designed and attached to the ligase ligands, achieving PROTAC precursors. Prior to PROTAC assembly, hydrogenolytic deprotection of the POI ligands and trifluoroacetic acid (TFA) deprotection of the precursor molecules were performed. A 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxide hexafluorophosphate (HATU)-mediated amide coupling of the two POI ligands with the corresponding deprotected PROTAC precursors yielded a set of six degraders.

Since PROTACs usually reside in the beyond Lipinski's rule of five (bRo5) chemical space,⁷ attention was paid to a first physicochemical and pharmacokinetic characterization of drug-likeness. The experimental distribution coefficient (*e*log*D*)^{211,212} and the percentage of compound bound to human serum albumin (plasma protein binding, PPB)²¹³ were determined *via* HPLC. Alkyl linker-based PROTACs demonstrated higher lipophilicity and plasma protein binding than their PEG-based counterparts. The inhibitory profile of the six PROTACs was delineated by measuring the *in vitro* formation of a 7-amino-4-methylcoumarin (AMC) fluorescent reporter upon trypsin cleavage of an HDAC isoenzyme-specific deacetylated substrate.^{214–216} Nanomolar to low micromolar HDAC6 inhibition was achieved with each PROTAC, whereas HDACs 1-4 were inhibited to less than 30% at a concentration of 30 μ M. However, the inhibitory potency of the hydroxamic acid vorinostat regarding HDAC6 was not outperformed. Following this, protein levels of HDAC6 in MM.1S multiple myeloma cells upon 24 hours treatment with 1 μ M of each PROTAC were analyzed by Western blot.²¹⁷ All six PROTACs exhibited a significant reduction of HDAC6 levels. The distinct degradation selectivity profile of the identified hit compounds of the CRBN and VHL subset were confirmed by Western blot analysis of HDAC1 and HDAC4 protein levels after 24 hours treatment at a PROTAC concentration of 1 μ M. For the affirmation of the hypothesized ternary complex-dependent degradation mechanism, competition experiments with both, the HDAC6 inhibitor vorinostat and the respective ligase ligand, which was either pomalidomide or VH298, were performed. Each competition approach preserved HDAC6 from PROTAC-induced proteolytic depletion. Pretreatment of MM.1S cells with the neddylation inhibitor MLN4924 also disabled UPS-mediated proteolysis of HDAC6. Finally, two tailored chemical negative controls of the hit compounds were synthesized by either introducing a methylated glutarimide portion²⁰⁸ or by the inclusion of an inversed stereogenic center in the hydroxyproline of the VHL ligand. In

turn, PROTAC molecules are incapable to adequately bind to the E3 ligases and eventually to promote degradation of HDAC6, thus further corroborating the proposed mechanism.

In conclusion, a set of six potent DFMO-based HDAC6 PROTACs was developed, addressing either the CRBN or VHL E3 ligase. Two representatives were selected for an in-depth characterization in order to give evidence for their selectivity profile and mode of action. These pave the way for the development of future non-hydroxamate PROTACs for clarifying the role of HDAC6 in various pharmacological pathways and diseases.

2.2 Author Contribution

Within this project, I designed, synthesized and structurally characterized compounds **1-6**, **18-20**, **22-27**, **29-31**, **33-35**, **37**, **38**, **41-48** and both chemical negative controls **1(-)** and **4(-)**, referring to the numeration within the publication. Apart from that, I contributed to the main text and the supporting information. Schemes 2 and 3 and Table 1 of the main text were prepared by myself.

3 Structure–Stability Relationship of NLRP3 Inflammasome-Inhibiting Sulfonylureas

Tim Keuler, Dominic Ferber, Michael Marleaux, Matthias Geyer and Michael Gütschow

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Please refer to Appendix II for the publication’s full text and supporting information.

3.1 Publication Summary

Sulfonylureas are mainly associated with antidiabetics, such as glyburide which is also referred to as glibenclamide. Representatives of this hypoglycemic drug class aim to initiate insulin secretion from beta cells of pancreas tissue, thus maintaining the blood glucose concentration of T2DM patients. In case of sulfonylureas, this is facilitated by blocking the tetrameric sulfonylurea receptor subunit of ATP-sensitive potassium ion channels. Disabled potassium ion efflux in turn causes membrane depolarization, followed by calcium ion influx and the opening of voltage-gated calcium channels, culminating in the secretion of insulin.²¹⁸

A phenotypic screening in 2001 revealed glibenclamide as a dose-dependent inhibitor of IL-1 β release from human monocytes with an IC₅₀ value of 12 μ M. Additional sulfonylureas and in particular diaryl sulfonylureas were found to exhibit nanomolar inhibitory potencies of IL-1 β release.⁹⁷ Further research finally led to the development of MCC950,^{98,219} which was disclosed as a highly potent NLRP3 and thus IL-1 β release inhibitor with an IC₅₀ value of 7.5 nM from mice BMDMs.⁹⁹ Since then, increasing effort was devoted to the identification of MCC950 relatives including the characteristic diaryl sulfonylurea moiety. Nevertheless, no in-depth reports on their chemical stability exist. Hence, a systematic investigation of the influence of substitution patterns on the chemical stability of sulfonylureas was performed.

In contrast to the *N*-alkyl and *S*-aryl substitution pattern in established antidiabetics, in this study particular emphasis was dedicated to the chemical stability of *N,S*-diaryl substituted analogs of the MCC950 scaffold. Assembly of all sulfonylureas required sodium hydride-promoted deprotonation of a sulfonamide and subsequent addition of an isocyanate. The applied isocyanates were either commercially available or *in situ* prepared from their respective amine precursors by means of a bis-(trichloromethyl) carbonate (BTC, triphosgene)-containing protocol. Six sulfonylureas were constructed from 1,2,3,5,6,7-hexahydro-*s*-indacen-4-amine, which could be obtained following slightly adapted reported procedures.^{219,220} The bulky

1,2,3,4,5,6,7,8-octahydroanthracen-9-amine, an indacene relative with faint resemblance, was synthesized *via* a two-step hydrogenation of 9-nitroanthracene under palladium and rhodium catalysis.²²¹ In order to obtain tailored sulfonamides for the positional scanning at the eastern part of the sulfonylurea scaffold, a reported multistep procedure towards 4-(2-hydroxypropan-2-yl)furan-2-sulfonamide was applied.^{219,222} An adapted protocol was employed for the synthesis of the thiophene analog. The final set of twelve sulfonylureas included ten diaryl sulfonylureas, one *N*-alkyl and one *S*-alkyl aryl sulfonylurea. In the next step, compounds were subjected to a HPLC-based chemical stability assay. Prior to injection, compounds were incubated in buffers at five different *pH* conditions (*pH* 1.0 to 8.4) for eight hours. All sulfonylureas, except for the glibenclamide fragment, exhibited decreased areas under the curve (AUCs) at *pH* 1 and 4. By referring to an internal standard, half-lives were calculated as a measure of decomposition. Incubation in buffers approximating or extending *pH* values of a neutral milieu (*pH* 6.0 to *pH* 8.4), rescued all compounds from decomposition. This attributes an increased chemical stability to their corresponding deprotonated anions.

By comparing half-lives of the compounds at harsh decomposition conditions (*pH* 1.0), bulky *N*-substituents, *i.e.* hexahydro-*s*-indacene and octahydroanthracene, surprisingly appeared to have destabilizing effects in contrast to their phenyl substituted counterparts. In addition, electron-withdrawing *N*-substituents also destabilized the compounds, whereas the electron-donating *N*-substituent in the glibenclamide fragment had a stabilizing impact. Intriguingly, on the eastern part of the molecules, a stabilizing effect was ascribed to the tertiary alcohol group in MCC950 and its thiophene analog.^{223,224} Both demonstrated increased half-lives compared to their close relatives lacking the tertiary alcohol moiety. A hypothesized decomposition reaction, including a decarboxylation, was substantiated with spiking experiments.

Four sulfonylureas were then subjected to SPR measurements to evaluate their binding affinity to human NLRP3. This experimental setting involves the immobilization of the recombinant biotinylated human NLRP3-NACHT domain on a streptavidin-functionalized sensor chip. MCC950 and its thiophene relative demonstrated similarly low dissociation constants, referring to high affinity binding. In contrast, their analogs with a phenyl *S*-substitution and an octahydroanthracene *N*-substitution pattern demonstrated poor or even complete loss of binding affinity to NLRP3. These results prove the hexahydro-*s*-indacene and the furan/thiophene-propanol moieties, sandwiching the sulfonylurea core, to be favorable for high affinity binding to human NLRP3. Thus, the pharmacophore of diaryl sulfonylureas targeting NLRP3 allows for changes, even though subtle modifications can drastically reduce binding affinity.

In summary, unexpected structure-stability relationships of sulfonylureas were observed. This valuable knowledge can be used for the future design of NLRP3 ligands or the handling of diaryl sulfonylureas in general.

3.2 Author Contribution

Within this project, I designed, synthesized and structurally characterized compounds **1-12, 14-18, 20-22, 24-26, 28, 30** and **31**, referring to the numeration within the publication. Apart from that, I contributed to the main text and the supporting information. Scheme 1 and Table 1 of the main text were prepared by myself.

4 Development of Fluorescent and Biotin Probes Targeting NLRP3

Tim Keuler,* Karl Gatterdam,* Anil Akbal, Marta Lovotti, Michael Marleaux, Matthias Geyer, Eicke Latz and Michael Gütschow

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*These authors contributed equally.

Please refer to Appendix III for the publication's full text and supporting information.

4.1 Publication Summary

Covalent and non-covalent probes have proven of great value as pharmacological tool compounds, *e.g.* for the identification of ligand binding sites or the cellular localization and quantitative determination of proteins. Fluorescence labeling of proteins is a commonly utilized method to visualize the envisaged protein within an intact cell. This can either be accomplished by the use of engineered fluorescent fusion proteins,^{58,225} or by the application of chemical probes. The latter allows for the detection of the native protein. In this field, coumarins are frequently introduced, because of their sufficient chemical and enzymatic stability as well as their good spectroscopic properties. The scaffold of the prototypic 7-aminocoumarins can be further improved by enhancing the rigidity of the anilinic group, thus providing appropriate quantum yields in aqueous media.²²⁶ The labeling of proteins with a biotinylated probe benefits from the strong interaction between biotin (vitamin B₇) and the proteins avidin, streptavidin and neutravidin. This technique enables targeted protein pull-down.²²⁷

Focus of this publication was the development and application of NLRP3 probes harnessing a fluorescent and rigid coumarin 343 or biotin reporter unit. MCC950 was chosen as an appropriate template, in order to establish an exit vector strategy, while retaining adequate binding affinity to NLRP3. Reports on the structure-activity relationships of NLRP3 inhibitors of the diaryl sulfonylurea type suggested to conserve the western hexahydro-*s*-indacene moiety. Modifications on the eastern part of the diaryl sulfonylurea group were rather accepted.^{105,108,228} In consideration of these rules, a Boc-protected *p*-(aminomethyl)benzenesulfonamide was used for the design of the expandable NLRP3 ligand. The study aimed to design a pair of both fluorescent and biotin probes. For the shorter probes, the respective reporter unit was immediately attached to the deprotected NLRP3 ligand. Two probes included a linker to bridge

the NLRP3 ligand and the reporter moiety. An orthogonally protected amino to carboxy linker was obtained by installation of a benzyloxycarbonyl (Cbz) protecting group at the terminal amine of 2-aminoethoxyethanol. Alkylation with *tert*-butyl bromoacetate at the terminal alcohol implemented a chain elongation with a protected carboxyl group.²²⁹ Coumarin 343 was synthesized following a reported procedure.^{230,231} Due to observed instability issues during the synthesis of diaryl sulfonylurea-containing compounds, the reporter units coumarin 343 and biotin were first attached to the mono-deprotected linker. This was either achieved by HATU or a *N*-hydroxysuccinimide (NHS)-promoted amide coupling.^{231,232} The two elongated probes were finally assembled *via* HATU-coupling. The appendage of the reporter units straight to the deprotected NLRP3 ligand in case of the shorter probes, was facilitated analogously.

SPR measurements of the four probes revealed distinct reduction of affinity to human NLRP3 in comparison to MCC950. This is deduced from the drastic change of the eastern part of the NLRP3 ligand. It is noteworthy that the rather small probes exhibited lower kinetic dissociation constants (K_D) than their elongated relatives, implying a tighter binding behavior. A dual step injection experiment of MCC950 followed by one of the shorter probes or *vice versa* showed just a negligible increase in response units. This finding indicates identical binding sites for the analytes and MCC950.

By means of a homogenous time-resolved fluorescence (HTRF) assay, IL-1 β release from immortalized murine BMDMs was measured as a result of inflammasome formation. Cells were primed with LPS and either stimulated with nigericin or with poly(deoxyadenylic-deoxythymidylic) acid, activating the NLRP3 or the AIM2 inflammasome, respectively. Both fluorescent probes completely blocked nigericin-induced IL-1 β release at concentrations of 2.5 μ M for the shorter and 10 μ M for the elongated compound. The two biotin probes instead lacked significant inhibition at 20 μ M. The extent of pyroptosis was determined by measuring the concentration of released cytosolic lactate dehydrogenase (LDH). In accordance with the previous observations, MCC950 and the shorter fluorescent probe rescued from NLRP3-related cell death at a concentration of 2.5 μ M. The AIM2 inflammasome-dependent LDH release by contrast was not prevented.

Immortalized murine BMDMs overexpressing a NLRP3-mCitrine fusion protein were used for the evaluation of the short fluorescent probe in confocal microscopy. By the use of appropriate excitation and emission wavelengths of either coumarin 343 or the fluorescent protein mCitrine confocal images were obtained. An overlay confirmed colocalization of the probe-dependent staining of NLRP3 and the fluorescent fusion protein.

Taken together, the application of the developed probes from this project shed light on large diaryl sulfonylurea NLRP3 ligands. These findings strengthen the purpose of installing an exit vector at the eastern part of the diaryl sulfonylurea core, without losing cell permeability and excessive target affinity.

4.2 Author Contribution

Within this project, I designed, synthesized and structurally characterized compounds **1-4, 6, 8-10, 12-14, 17, 18, 20** and **21**, referring to the numeration within the publication. Apart from that, I contributed to the main text and the supporting information. Schemes 1 and 2 and Figures 1 and 2 of the main text were prepared by myself.

5 Development of Chimeric NLRP3 Ligands

5.1 Development of NLRP3-targeting PROTACs

This project aimed at the development of NLRP3-targeting PROTACs. As molecules of this innovative drug modality consist of three parts, *i.e.* the POI ligand, a linker portion and an E3 ligase ligand, building blocks were individually synthesized and finally assembled. For achieving structural diversity in order to obtain active PROTACs, different NLRP3 ligands, varying linkers and E3 ligase ligands were combined.

5.1.1 Synthesis of NLRP3 Ligands Including Different Exit Vectors

For the proposed PROTAC design, MCC950 served as a template for the POI ligand. Its molecular structure is dominated by a central sulfonylurea moiety, which is flanked by a western and an eastern substituent. MCC950 and its relatives coined the design of future diaryl sulfonylurea NLRP3 inhibitors. Hence, they commonly retain the characteristic butterfly-shaped 1,2,3,5,6,7-hexahydro-*s*-indacene group as the western substituent (red, Figure 13). This initial tendency was furthermore corroborated by crystal structures of human NLRP3 in complex with MCC950.⁵² Although the 2-(furan-3-yl)propan-2-ol residue on the eastern part of MCC950 (Figure 7) ensures high affinity to NLRP3, it was shown to be exchangeable.^{105,108,228} Apart from structural diversified inhibitors,^{104,105} also the introduction of large eastern substituents (green, Figure 13) at the sulfonylurea group were allowed, *e.g.* in order to achieve photoaffinity probes.^{100,101} By taking these structure-activity relationships into account, four NLRP3 ligands implementing different exit vector strategies were conceived.

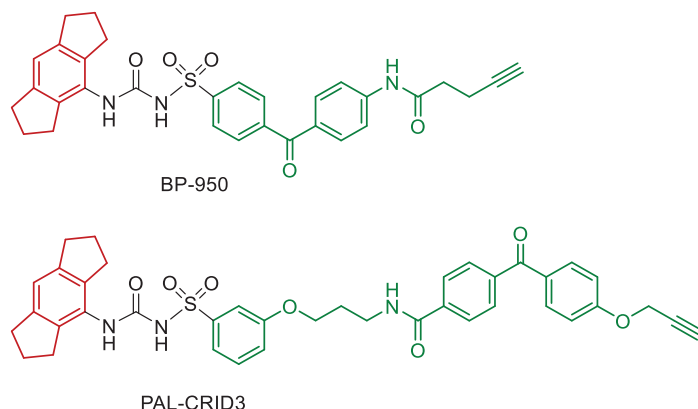


Figure 13. Photoaffinity probes for irreversible crosslinking to NLRP3.

In the past, the functional group of sulfonylureas was mainly associated with antidiabetics (*e.g.* carbutamide) and herbicides for agricultural use (*e.g.* chlorsulfuron, Figure 14).^{218,233} The methods for sulfonylurea synthesis can be discriminated according to the applied starting materials. One approach is the reaction of sulfonamides with appropriate carbon-centered electrophiles. By this, the formation of a new carbon-nitrogen bond is induced, yielding the sulfonylurea moiety. For this purpose, isocyanates or activated carbamoyl derivatives such as carbamates are frequently applied.²³⁴

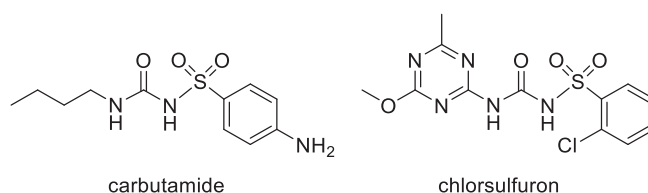


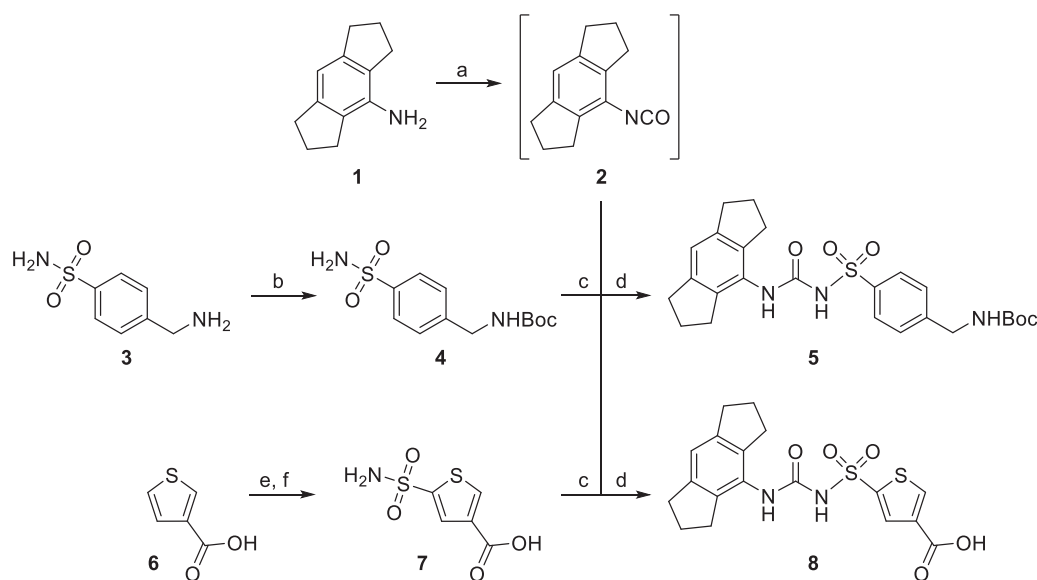
Figure 14. The antidiabetic drug carbutamide and the herbicide chlorsulfuron hold a sulfonylurea group.

Prior to the reaction with an electrophile, sulfonamides can be deprotonated in a salification step, using a base, *e.g.* NaH,^{108,222,228} NaOH¹⁰¹ or sodium methoxide.²¹⁹ Other studies reported a CuCl-catalyzed procedure for direct carbon-nitrogen bond formation between sulfonamide and isocyanate, in order to avoid basic reaction conditions.²³⁵ Since many amines but less isocyanates or carbamates are commercially available, robust protocols for the transformation of amines into these carbon-centered electrophiles are needed. Such procedures include the di-*tert*-butyl dicarbonate (Boc₂O)-dependent and *N,N*-dimethylpyridin-4-amine (DMAP)-catalyzed conversion of both alkyl and aryl amines into their corresponding isocyanates.^{222,228,236} Although the Boc₂O/DMAP protocol takes advantage from less hazardous reagents compared to the use of highly toxic phosgene or its close relative triphosgene for isocyanation, it failed in my hands. In addition to these straightforward methodologies in case of amine starting materials, alternative mechanisms have been reported for deviating starting reagents. These include the *in situ* formation of isocyanates as a result of Curtius rearrangement of intermediate acyl azides obtained from carboxylic acids.^{234,237}

Apart from isocyanation of amines, carbamates as alternate electrophiles are commonly prepared, *e.g.* by using diphenyl carbonate²³⁸ or diverse chloroformates as carbonyl source. Upon treatment with organic bases at elevated temperatures, carbamates form their corresponding isocyanates.²³⁴

For the synthesis of tailored NLRP3 ligands, 1,2,3,5,6,7-hexahydro-*s*-indacen-4-amine (**1**) was initially transformed into the corresponding carbamate by using 4-chlorophenyl chloroformate. Since this approach and the application of the Boc₂O/DMAP protocol appeared to be less practicable, triphosgene was used for isocyanation. By dropwise addition of the amine to an equimolar mixture of triphosgene in dry tetrahydrofuran (THF) in the presence of triethylamine (TEA), the formation of a symmetric urea was avoided. Heating to reflux and a nonaqueous workup by means of filtration yielded isocyanate **2** (Scheme 1), which was used in the next step without further purification. Isocyanate **2** was always freshly prepared ahead of sulfonylurea formation.

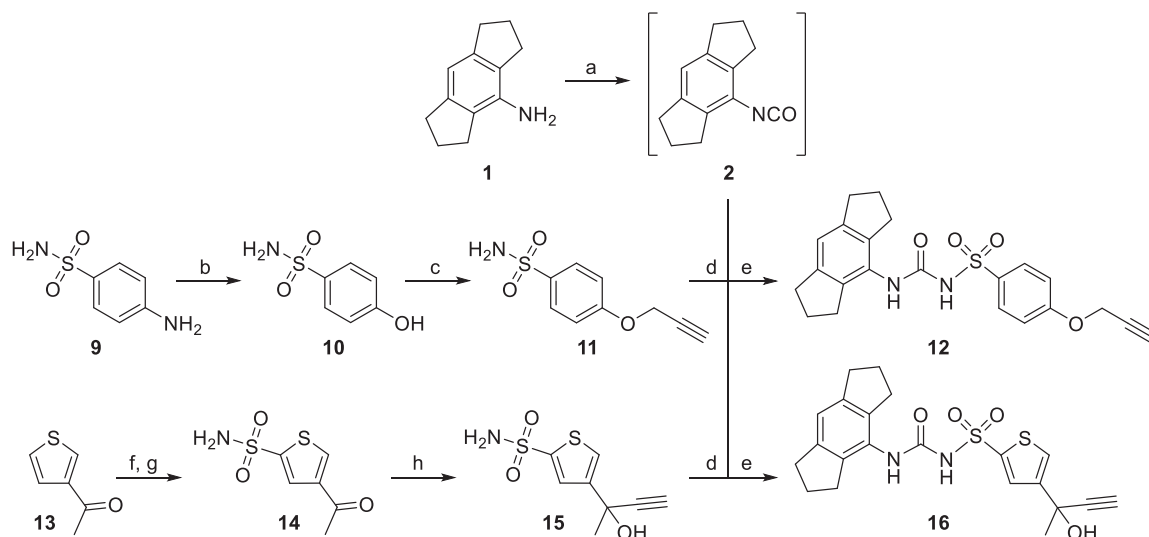
For NLRP3 ligand diversification, various sulfonamides were deprotonated with NaH in THF and subsequently reacted with isocyanate **2** to afford the corresponding NLRP3 ligands (Schemes 1 and 2). Prior to this, sulfonamides had to be prepared. Treatment of sulfonamide **3** with Boc₂O yielded sulfonamide **4**, bearing a Boc-protected amino function. Thiophene building block **6** was transformed into sulfonamide **7** by a chlorosulfonylation and subsequent ammonolysis with ammonium hydroxide solution.²³⁹ Sulfonamides **4** and **7** were then each reacted with isocyanate **2** to give their corresponding sulfonylureas **5** and **8**. These can either be used for the attachment of terminal carboxy or amino linkers *via* amide coupling reactions.



Scheme 1. Design of NLRP3 ligands for subsequent amide coupling reactions.

Reagents and conditions: (a) BTC, TEA, THF, 0 °C for 30 min, rt for 30 min, 75 °C for 30 min; (b) Boc₂O, TEA, MeCN, 0 °C to rt, 18 h; (c) NaH, THF, 0 °C, 30 min; (d) THF, rt, 2–4 h; (e) ClSO₃H, 0 °C for 30 min, 60 °C for 3 h; (f) NH₄OH, MeCN, 50 °C, 1 h.

To expand the possibilities for linker attachment *via* copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC) as a version of Huisgen cycloaddition, two NLRP3 ligands with a terminal alkyne functionality were designed (Scheme 2). Sulfanilamide (**9**) was diazotized with NaNO_2 in the presence of concentrated H_2SO_4 and H_2O and subsequently transformed into phenol **10** at elevated temperatures.²⁴⁰ The following alkylation with propargyl bromide afforded sulfonamide **11**.²⁴¹ Another sulfonamide **14** was obtained from 3-acetylthiophene (**13**) through chlorosulfonylation and subsequent ammonolysis with ammonia gas. A Grignard reaction at the carbonyl moiety of **14** with ethynylmagnesium bromide in THF delivered the tertiary alcohol **15** under racemization. Again, deprotonation of **11** and **15** with NaH in THF and respective addition of isocyanate **2** yielded the corresponding NLRP3 ligands **12** and **16**.



Scheme 2. Design of NLRP3 ligands for subsequent CuAAC.

Reagents and conditions: (a) BTC, TEA, THF, 0 °C for 30 min, rt for 30 min, 75 °C for 30 min; (b) NaNO_2 , H_2SO_4 , H_2O , 0 °C, 90 °C for 2 h; (c) K_2CO_3 , propargyl bromide, DMF, rt, 18 h; (d) NaH, THF, 0 °C, 30 min; (e) THF, rt, 2-4 h; (f) ClSO_3H , 0 °C for 30 min, 60 °C for 3 h; (g) NH_3 (1 atm), MeCN, 1 h; (h) ethynylmagnesium bromide, THF, 0 °C, rt for 2 h.

By these synthetic routes, the four NLRP3 ligands **5**, **8**, **12** and **16** with diverse functionalities for coupling reactions to adequately functionalized linker tethers were obtained. Among these, especially diaryl sulfonylurea **16**, conserving the tertiary alcohol as a feature of MCC950, was expected to be promising in terms of high NLRP3 affinity.

5.1.2 Synthesis of E3 Ligase Ligands

For the successful recruitment of E3 ligases, appropriate ligands including a functionality for linker attachment were needed. Three well-established ligands for addressing the E3 ligases cereblon,^{23,242} VHL^{209,210} and IAP (inhibitor of apoptosis protein)²⁴³ were synthesized in accordance with literature. As a part of chimeric degraders, all ligands have proven of great value in recruiting their corresponding E3 ligase.

In 2015, the first PROTACs recruiting the E3 ligase CRBN with the aid of IMiDs thalidomide²² and pomalidomide²³ were reported. Since then, much effort has been dedicated towards the exploration of new CRBN ligands, in order to enhance binding affinity²⁴⁴ and to overcome reported stability issues concerning the phthalimide and glutarimide rings.^{245,246}

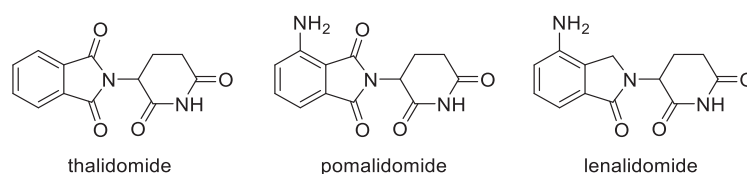
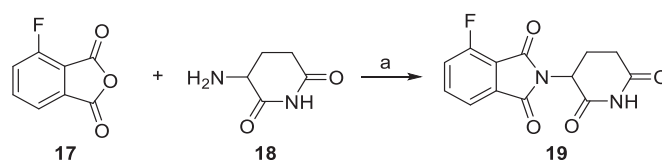


Figure 15. IMiDs as a common template for the recruitment of the E3 ligase cereblon.

However, for initial evaluation of NLRP3 to be susceptible to PROTAC-induced proteolytic degradation, pomalidomide was chosen as CRBN-addressing ligand (Figure 15). For this purpose, 3-fluorophthalic anhydride (**17**) was refluxed together with the hydrochloride of 3-aminopiperidine-2,6-dione (**18**) in the presence of AcOH to obtain 4-fluoro-thalidomide (**19**) (Scheme 3).^{208,247,248} Imide **19** is equipped with a fluorine allowing for a nucleophilic aromatic substitution reaction (S_NAr) with an amino-functionalized linker, in turn generating the pomalidomide portion.^{242,249} In addition, multiple alternative approaches to attach linkers to the thalidomide core have been reported, *e.g.* by means of Sonogashira coupling, Suzuki coupling or a Mitsunobu reaction.²⁵⁰



Scheme 3. Synthesis of the CRBN ligand 4-fluoro-thalidomide (**19**).

Reagents and conditions: (a) NaOAc, AcOH, reflux, 4 h.

The second E3 ligase to be exploited was VHL. The earlier used HIF1 α -mimicking amino acid sequence (ALAPYIP) required enzymatic hydroxylation of the central proline to act as the VHL E3 recognition domain,¹³ which was corroborated in the course of conformational analyses.²⁵¹ In 2012, structural optimizations were conducted on both sides of a central hydroxyproline core and eventually led to the identification of small molecular ligands.^{25,26,252} Filling of the so-called right-hand side (RHS) of the VHL/HIF1 α interaction surface was achieved by introducing a benzylamine moiety with a terminal methylthiazole at the carboxy function of the hydroxyproline core.^{25,253} Derivatizing the N-terminal left-hand side (LHS) of this pharmacophore with a *tert*-leucine (Tle) moiety further improved binding behavior towards VHL, as confirmed by ITC.²⁷ The obtained N-terminal acetylated inhibitor is commonly referred to as VH032 and serves as a prototypic VHL ligase ligand (Figure 16).^{27,254} First PROTACs including a small molecular VHL ligase ligand while targeting endogenous substrates were reported in 2015.^{11,29} Later on, the VHL binding motif was modified at the benzylamine portion by introducing a methyl group (Me-VH032), yielding an additional *S*-configured stereocenter in comparison to VH032 (Figure 16).^{255,256} Incorporated into PROTACs,^{209,210,255,257} the methyl derivative exhibited increased degrading potency than PROTAC analogs missing the methyl group.²⁵⁶

Application of this small molecular pharmacophore for ligase recruitment in PROTAC design requires an exit vector for linker attachment, while various positions at the VHL ligand are accessible. The most common variants constitute conjugation of a linker at the terminal amino function of the LHS substituent,²⁵⁶ or at a phenolic group of the RHS substituent.^{28,258} Less studies reported the implementation of a linker junction either at the RHS benzylic methylene moiety²⁵⁵ or by installing a thioether at the sidechain residue of the LHS substituent.^{30,259} A recent study reported a novel exit vector strategy at the RHS thiazole moiety.²⁶⁰

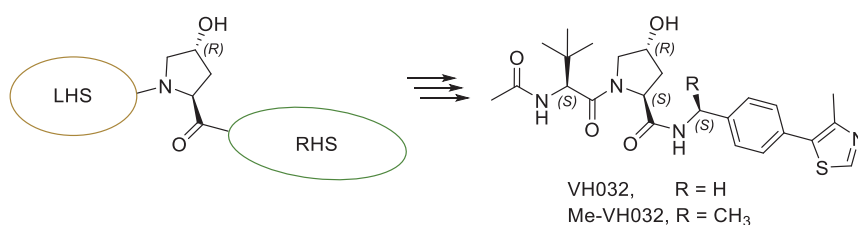
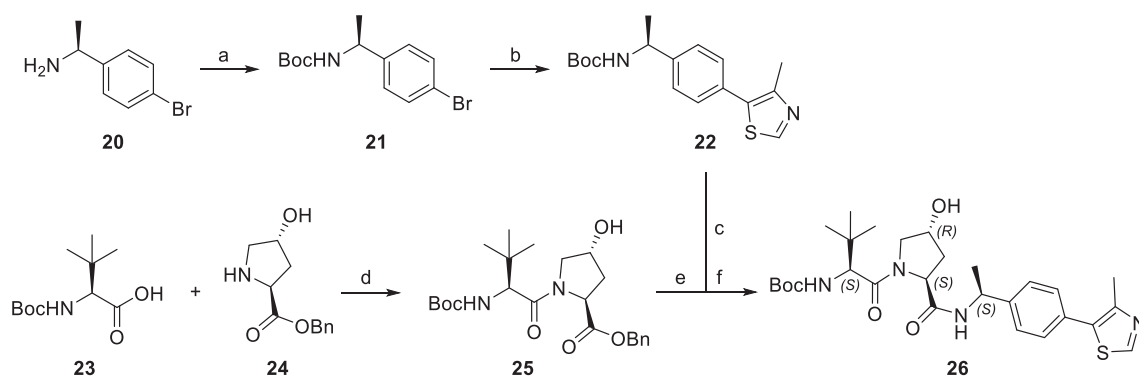


Figure 16. Development of VH032 as a small molecular VHL ligand. A central hydroxyproline core is substituted at the LHS and RHS of the VHL/HIF1 α interaction surface.

In this study, an analog of VH032, bearing the described methyl group, was prepared in order to attach a carboxy terminal linker to the amino group of *tert*-leucine as the LHS substituent. The convergent synthesis was performed similar to previous reports (Scheme 4).^{209,210,255} First, amino building block **20** was Boc-protected (**21**)²¹⁰ and subsequently coupled with 4-methylthiazole under Heck conditions²⁶¹ yielding **22**.²¹⁰ In the second branch, dipeptide **25** was synthesized from Boc-Tle-OH (**23**) and monoprotected hydroxyproline (**24**) via a HATU-promoted amide coupling. After TFA-mediated deprotection of **22** and hydrogenolytic cleavage of benzyl ester **25**, another amide coupling afforded the Boc-protected VHL ligand **26**.^{209,210,255}



Scheme 4. Convergent synthesis of the Boc-protected VHL ligand **26**.

Reagents and conditions: (a) NaHCO₃, Boc₂O, H₂O, EtOAc, 0 °C, 2 h; (b) Pd(OAc)₂, KOAc, 4-methylthiazole, DMA, 130 °C, 4 h; (c) TFA, CH₂Cl₂, rt, 2 h; (d) HATU, DIPEA, DMF, 0 °C, rt for 18 h; (e) Pd/C, H₂ (1 atm), EtOH, rt, 18 h; (f) HATU, DIPEA, DMF, rt, 18 h.

Another group of noticeable proteins are individual members of the inhibitor of apoptosis protein (IAP) family. From these, especially the two cellular inhibitor of apoptosis proteins 1 and 2 (cIAP1; cIAP2) and the X-chromosome linked IAP (XIAP) gained attraction in the field of TPD. They include a really interesting new gene (RING) domain with ubiquitin ligase activity beside three BIR domains.²⁶² A derivative of the immunomodulatory drug bestatin, *i.e.* methyl bestatin, was found to interact with the BIR domain of cIAP1. By this, cIAP1 was ubiquitinated by its own RING domain eventually causing UPS-dependent degradation.¹⁸ This finding led to the concept of IAP-dependent protein knockdown of CRABPs *via* a chemical knockdown approach, based on methyl bestatin.^{17,18} Heterobifunctional molecules utilizing IAPs as E3 ligases for TPD were thus termed specific and non-genetic IAP-dependent protein erasers (SNIPERs).^{263,264} Since bestatin and the corresponding SNIPERS may be disadvantageous due to moderate binding affinity and autoubiquitination of cIAP1,¹⁸ alternative IAP ligands are appreciated.²⁶⁵

The endogenous IAP inhibiting protein second mitochondria derived activator of caspases/direct IAP binding protein with low pI (Smac/DIABLO)^{266,267} laid the foundation for future inhibitor design. Hence, the resulting so-called Smac peptidomimetics (Smac-mimetics) imitate the N-terminal tetrapeptide (AVPI) of Smac/DIABLO (Figure 17).^{268–270} One early tetrapeptide was MV1 (Figure 17), which was incorporated into a CRABP-targeting PROTAC, while addressing cIAP1 as E3 ligase. A dose-dependent degradation of the POI proved the MV1 moiety to be a sufficient IAP ligand.²⁷¹ Many efforts have been devoted to the elucidation of structure-activity relationships, based on AVPI. These include introduction of non-natural substituents, various cyclization strategies or the generation of bivalent molecules.²⁷² Among these agents was compound 11 (Figure 17), which showed high binding affinity to the BIR3 domain of XIAP.²⁷³ Some advanced Smac-mimetics entered clinical trials, as they represent a promising approach to restore caspase activation and consecutive apoptosis in various cancer diseases through IAP inhibition.²⁷² For the envisaged design of IAP addressing NLRP3 degraders, compound 11 served as a template, since it was used in a PROTAC approach several times.^{243,274–277}

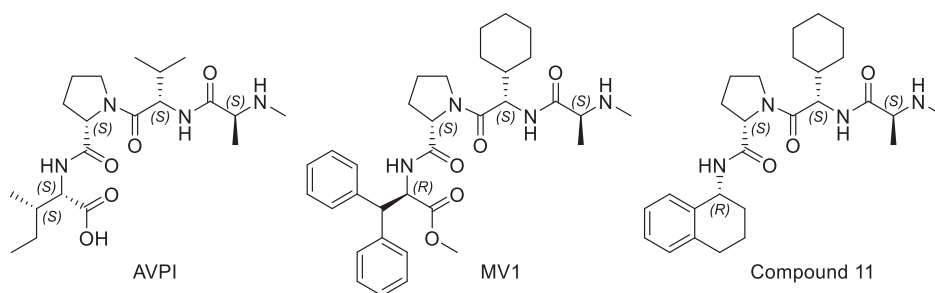
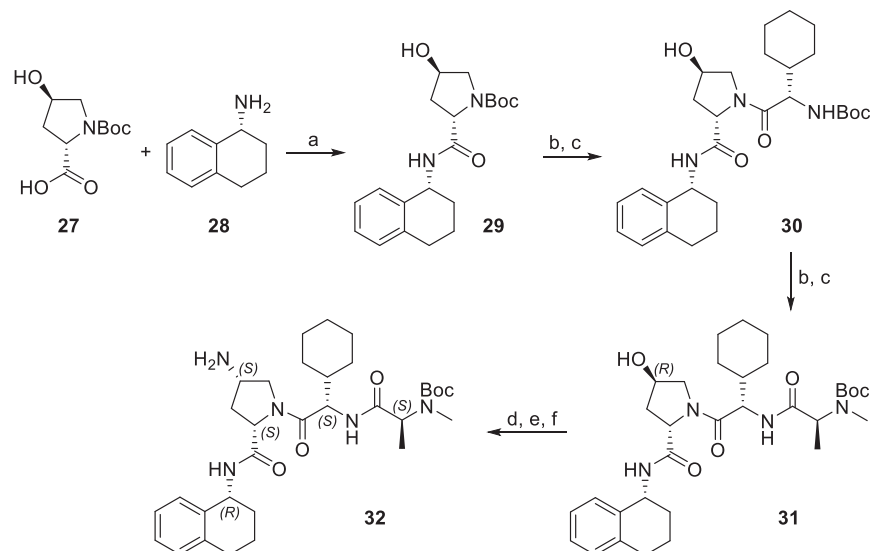


Figure 17. Development of SMAC-mimetics.

The starting point for IAP ligand synthesis (Scheme 5) was the 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC $\times$ HCl)-mediated amide coupling of hydroxyproline **27** with amine **28**. Then, Boc-protected dipeptide **29** was deprotected with HCl in EtOAc (ethyl acetate). The intermediate salt was subsequently subjected to an amide coupling reaction with Boc-L-cyclohexylglycine to afford **30**. Deprotection of **30**, and a further amide coupling with Boc-N-methyl-L-alanine yielded Boc-protected IAP ligand **31**, as described before.^{274,278} For the attachment of linkers, the hydroxyl function at the *R*-configured stereocenter of the hydroxyproline core was transformed into an amine function *via* a three step one-pot protocol. First, alcohol **31** was converted into the corresponding methanesulfonate ester, followed by a second-order nucleophilic substitution (S_N2) reaction with NaN_3 . As a result, the *S*-configured azido derivative was obtained under Walden inversion of the

stereocenter. A Staudinger reduction of the intermediate azide yielded the amino-functionalized IAP ligand **32**. For proper binding to BIR3 domains of IAPs, the terminal Boc-protecting group at the alanine portion of **32** needs to be cleaved.



Scheme 5. Synthesis of the IAP ligand **32**.

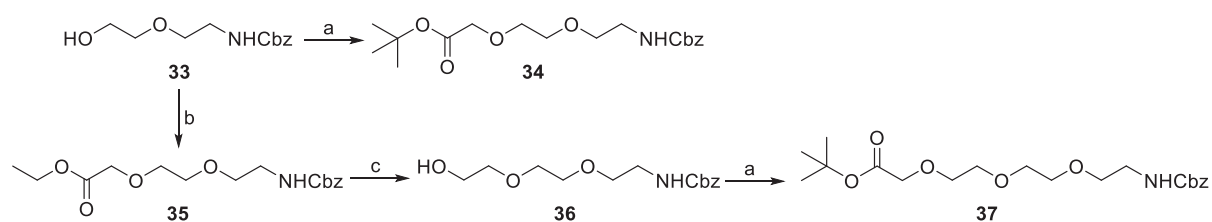
Reagents and conditions: (a) HOBt, TEA, EDC $\times$ HCl, CH₂Cl₂, 0 °C for 15 min, rt for 18 h; (b) 1 N HCl in EtOAc, rt, 18 h; (c) HOBt, DIPEA, EDC $\times$ HCl, DMF, 0 °C for 15 min, rt for 18 h; (d) TEA, MsCl, CH₂Cl₂, 0 °C, rt for 24 h; (e) NaN₃, DMF, 70 °C, 72 h; (f) PPh₃, THF, rt, 1 h, NH₄OH, rt, 18 h.

5.1.3 Synthesis of Functionalized Linker Building Blocks

For the combination of NLRP3 ligands with ligase ligands, a set of tailored α,ω -bifunctionalized linkers was necessitated. According to the exit vector functionalities of the designed ligands, this study focused on the development of linker molecules bearing terminal carboxy, amino and azido groups. In order to cover a broad spectrum of influencing factors, such as polarity, size and flexibility, PEG- as well as alkyl-based linkers or mixed hybrids thereof were generated.

5.1.3.1 Synthesis of Bifunctionalized Linkers for Amide Coupling Reactions

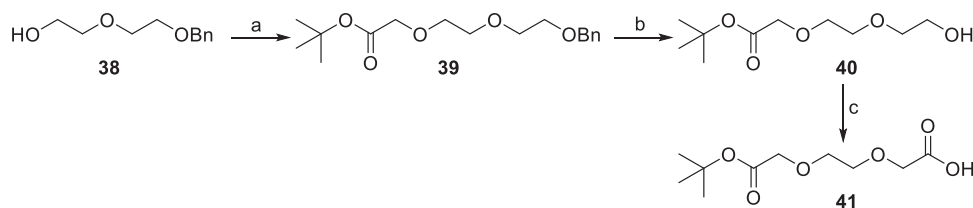
The synthesis of PEG-based carboxy-to-amino linkers was performed according to previously reported procedures.^{229,249,279} First, 2-(2-aminoethoxy)ethanol was Cbz-protected with benzyl chloroformate to yield **33** (Scheme 6). Its alcohol group was alkylated either with *tert*-butyl bromoacetate to give orthogonally protected linker **34** or ethyl bromoacetate to yield **35**. Ethyl ester **35** was reduced to alcohol **36** under NaBH₄ treatment. Subsequent alkylation with *tert*-butyl bromoacetate afforded the extended linker **37**.^{229,249}



Scheme 6. Synthesis of orthogonally protected carboxy to amino linkers.

Reagents and conditions: (a) KO*t*Bu, *tert*-butyl bromoacetate, THF, 0 °C for 3 h, rt for 18 h; (b) KO*t*Bu, *i*BuOH, ethyl bromoacetate, toluene, 0 °C for 3 h, rt for 18 h; (c) NaBH₄, THF, reflux for 15 min, MeOH, rt, reflux for 60 min.

The synthesis of PEG-based mono-protected carboxy-to-carboxy linkers was performed according to previously reported procedures, and employed on additional starting materials.^{259,280} For this purpose, mono-benzylated diol **38** was alkylated with *tert*-butyl bromoacetate to yield **39** (Scheme 7).²⁸⁰ Hydrogenolytic deprotection gave **40**, which was oxidized to carboxylic acid **41** via a (2,2,6,6-tetramethylpiperidin-1-yl)oxyl (TEMPO) and bis(acetoxy)iodobenzene (BAIB)-including protocol.²⁵⁹



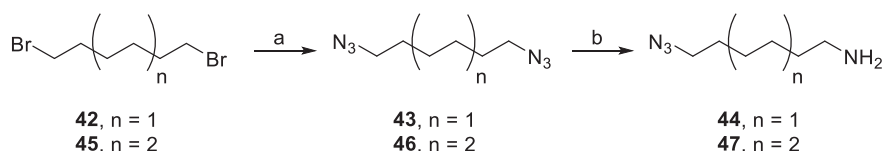
Scheme 7. Synthesis of the mono-protected carboxy to carboxy linker **41**.

Reagents and conditions: (a) KO^tBu, *tert*-butyl bromoacetate, THF, 0 °C for 1 h, rt for 18 h; (b) Pd/C, H₂ (1 atm), EtOH, rt, 18 h; (c) TEMPO, BAIB, MeCN, H₂O, rt, 16 h.

5.1.3.2 Synthesis of Bifunctionalized Linkers for CuAAC and Amide Coupling

A set of straightforward methods for the conjugation of molecules and/or biomolecules²⁸¹ is ‘click’ chemistry.²⁸² In particular, it is valuable for combinatorial chemistry attempts. Among these multiple techniques, CuAAC represents the most well-established method by reacting alkynes with azides under Cu(I)-catalysis.^{283–285} Since the alkyne portion is installed at the NLRP3 ligands, linkers with a terminal azido group were synthesized. To conjugate the outlined E3 ligase ligands to the linkers, a second terminal functionality in terms of an amino or carboxy group was implemented at each linker molecule.

Alkyl linkers of the azido-to-amino type were realized starting from α,ω -dibromoalkanes (**42**, **45**). These underwent azidolysis to give α,ω -diazidoalkanes **43** and **46** (Scheme 8). Staudinger reductions of **43** and **46** with one equivalent triphenylphosphine (PPh₃) each, achieved azido to amino linkers **44** and **47**.



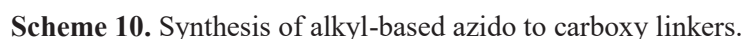
Scheme 8. Synthesis of alkyl-based azido to amino linkers.

Reagents and conditions: (a) NaN₃, DMF, 80 °C, 24 h; (b) PPh₃, H₂O, THF, rt, 18 h.

For the design of PEG-based linkers of the azido-to-amino type, diols of varying length were subjected to a reported three-step protocol (Schemes 9).²⁸⁶ First, both alcoholic groups of diols were converted to their corresponding tosylates (**49**, **53**, **57**, **61**, **65**). Second, azidolysis with NaN₃ gave their respective diazido linkers (**50**, **54**, **58**, **62**, **66**). Third, Staudinger reduction with equivalent amounts of PPh₃ afforded the PEG-based azido to amino linkers (**51**, **55**, **59**, **63**, **67**). The elongated diol **64** was synthesized beforehand by a Williamson ether synthesis, according to reported reaction conditions.²⁸⁷



For the attachment of amino-terminal E3 ligase ligands, *i.e.* the VHL and IAP ligands, carboxy-functionalized azido linkers were synthesized. Reacting ω -bromoalkanoic acids **68** and **70** with NaN₃ in dimethylformamide (DMF) at elevated temperature yielded alkyl-based azido-to-carboxy linkers **69** and **71** (Scheme 10).²⁸⁸

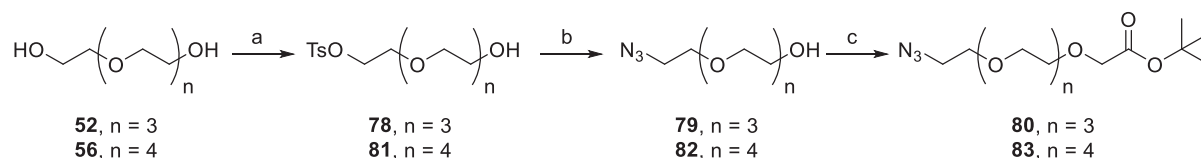


One approach to obtain PEG-based azido-to-carboxy linkers, was the azidolysis of chlorinated ethylene glycols (**72**, **75**) to their corresponding azides (**73**, **76**) (Scheme 11).²⁸⁹ Subsequent alkylation with *tert*-butyl bromoacetate under basic conditions in presence of tetrabutylammonium hydrogen sulfate (TBAHS) afforded *tert*-butyl protected azido to carboxy linkers **74** and **77**, as previously reported.²⁸⁹



42

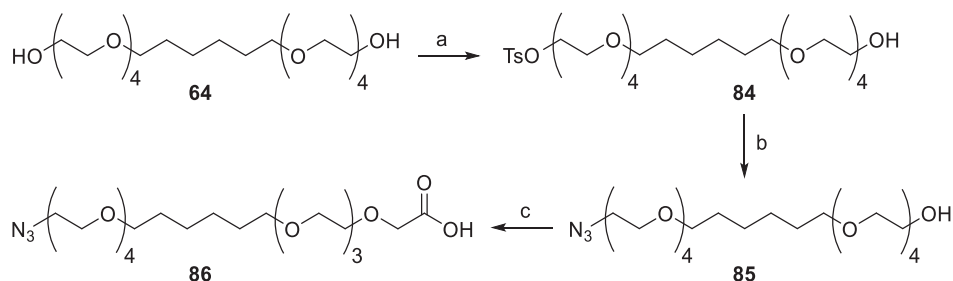
In case of longer linkers, the synthetic route started with ethylene glycols **52** and **56**, which were mono-tosylated to **78** and **81**, due to the use of excess diol (Scheme 12).²⁹⁰ A following azidolysis gave **79** and **82**. Alkylation extended the linkers to *tert*-butyl protected azido to carboxy linkers (**80**, **83**).



Scheme 12. Synthesis of PEG-based azido to carboxy linkers.

Reagents and conditions: (a) TEA, TsCl, CH₂Cl₂, 0 °C, rt for 18 h; (b) NaN₃, DMF, 80 °C, 24 h; (c) TBAHS, *tert*-butyl bromoacetate, 9.5 M NaOH (aq), toluene, 0 °C, rt for 22 h.

To achieve the direct analogs of the linkers within the azido to amino linker set, the counterpart of the long linker **67** was synthesized with a diverging final step (Scheme 13). First, diol **64** was mono-tosylated to **84** and subsequently converted to **85** through NaN₃ treatment. After work-up, **85** was carefully oxidized in an opened reaction vessel to obtain the long azido to carboxy linker **86**.



Scheme 13. Synthesis of the extended azido to carboxy linker **86**.

Reagents and conditions: (a) TEA, TsCl, CH₂Cl₂, 0 °C, rt for 18 h; (b) NaN₃, DMF, 80 °C, 24 h; (c) TEMPO, BAIB, MeCN, H₂O, rt, 16 h.

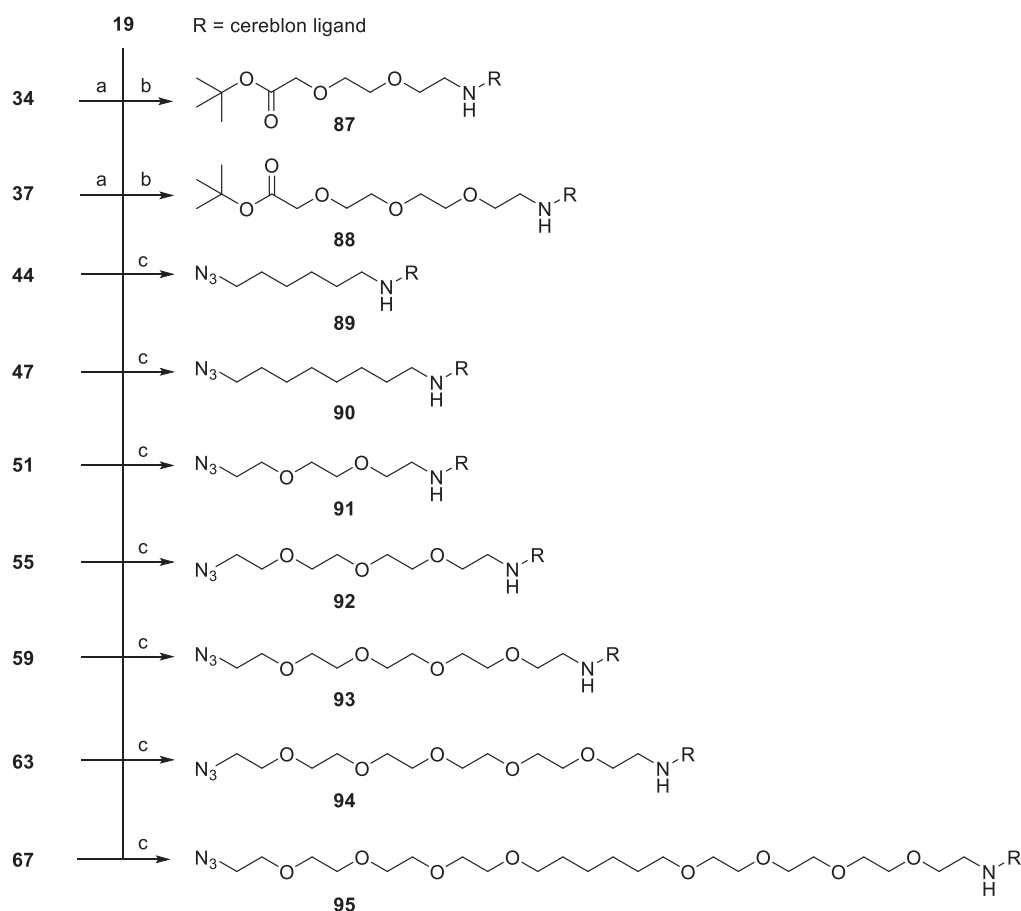
The herein used key reactions, including tosylation, azidolysis, selective Staudinger reduction, alkylation or TEMPO/BAIB oxidation, served as well applicable synthetic procedures. Hence, the prepared linker set finally comprised four different linker types: (i) carboxy-to-amino, (ii) carboxy-to-carboxy, (iii) azido-to-amino and (iv) azido-to-carboxy.

5.1.4 Synthesis of PROTAC Precursors

Since instability issues may be raised to the diaryl sulfonylurea moiety in NLRP3 ligands, their introduction into PROTAC molecules generally represented the last synthetic step. Hence, the E3 ligase ligands were first attached to the linkers.

5.1.4.1 Synthesis of PROTAC Precursors Addressing CRBN

Precursor molecules addressing CRBN were achieved by joining 4-fluoro-thalidomide (**19**) and amino-terminal linkers, in turn releasing a pomalidomide motif. These S_NAr reactions were performed in dipolar and aprotic solvents such as dimethylsulfoxide (DMSO)²⁴⁹ and DMF²⁴² in the presence of DIPEA (*N,N*-diisopropylethylamine) at elevated temperatures. For the synthesis of carboxy-terminal CRBN precursors (**87**, **88**), Cbz-protected linkers **34** and **37** were first cleaved by hydrogenolysis (Scheme 15). In order to obtain clickable azido-terminal cereblon-addressing precursors (**89-95**), linkers obtained from one-sided Staudinger reduction were also attached to 4-fluoro-thalidomide (**19**) via S_NAr under reported conditions (Scheme 14).²⁴²

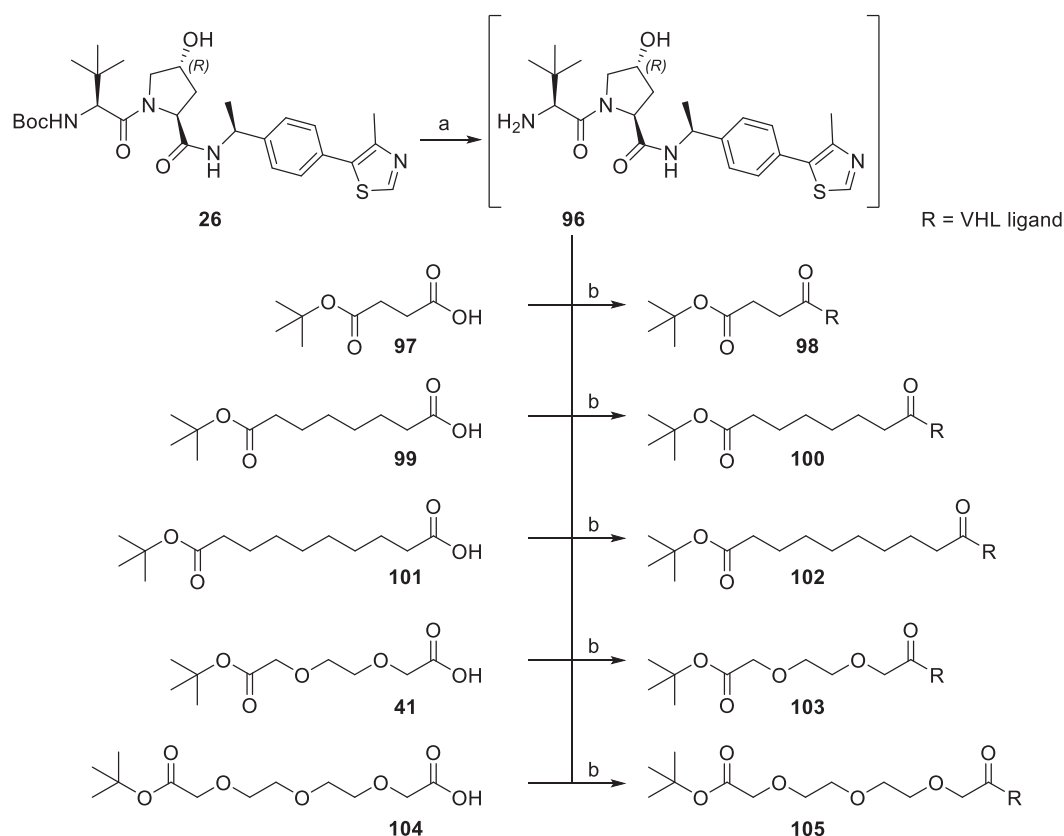


Scheme 14. Synthesis of *tert*-butyl protected carboxy to CRBN PROTAC precursors.

Reagents and conditions: (a) Pd/C, H₂ (1 atm), EtOAc, rt, 18 h; (b) DIPEA, DMSO, 90 °C, 18 h; (c) DIPEA, DMF, 90 °C, 4 h.

5.1.4.2 Synthesis of PROTAC Precursors Addressing VHL or IAP

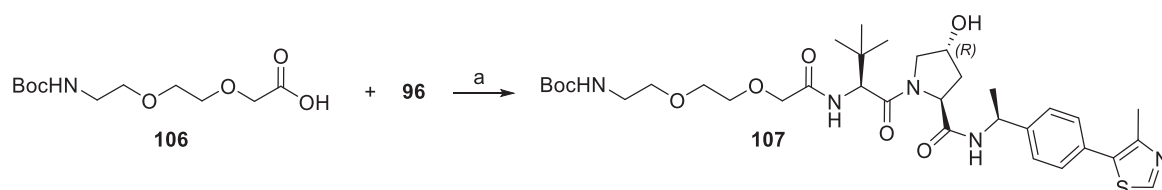
As outlined, linker attachment for precursor molecules towards the E3 ligase VHL was realized at the terminal amino function of the LHS substituent of **26**. For this purpose, Boc-protected VHL ligand **26** was deprotected with TFA prior to each linker appendage. A HATU-mediated amide coupling of the mono-protected dicarboxylic acids with deprotected VHL ligand **96** yielded *tert*-butyl protected carboxy-terminal VHL-addressing precursors (Scheme 15).



Scheme 15. Synthesis of *tert*-butyl protected carboxy to VHL PROTAC precursors.

Reagents and conditions: (a) TFA, CH₂Cl₂, rt, 2 h; (b) HATU, DIPEA, DMF, rt, 18 h.

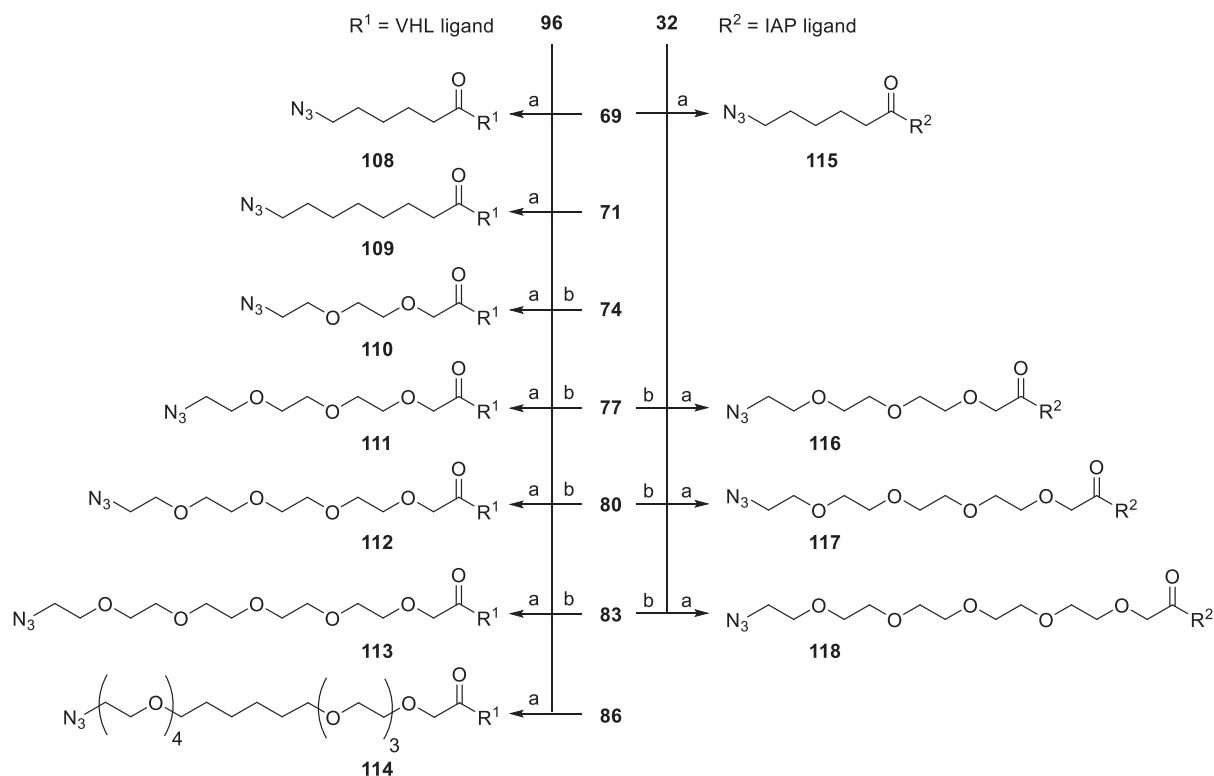
One individual heterobifunctional linker (**106**) was coupled with deprotected VHL ligand **96** in order to obtain the Boc-protected amino terminal VHL-addressing precursor **107** (Scheme 16).



Scheme 16. Synthesis of the Boc-protected amino to VHL PROTAC precursor **107**.

Reagents and conditions: (a) HATU, DIPEA, DMF, rt, 18 h.

The preparation of azido-terminal VHL or IAP-addressing precursors was analogously conducted by using azido to carboxy linkers and deprotected VHL ligand **96** or IAP ligand **32** respectively. In the case of linkers being prepared *via* a final alkylation step, *tert*-butyl esters were cleaved under TFA treatment prior to amide coupling (Scheme 17).



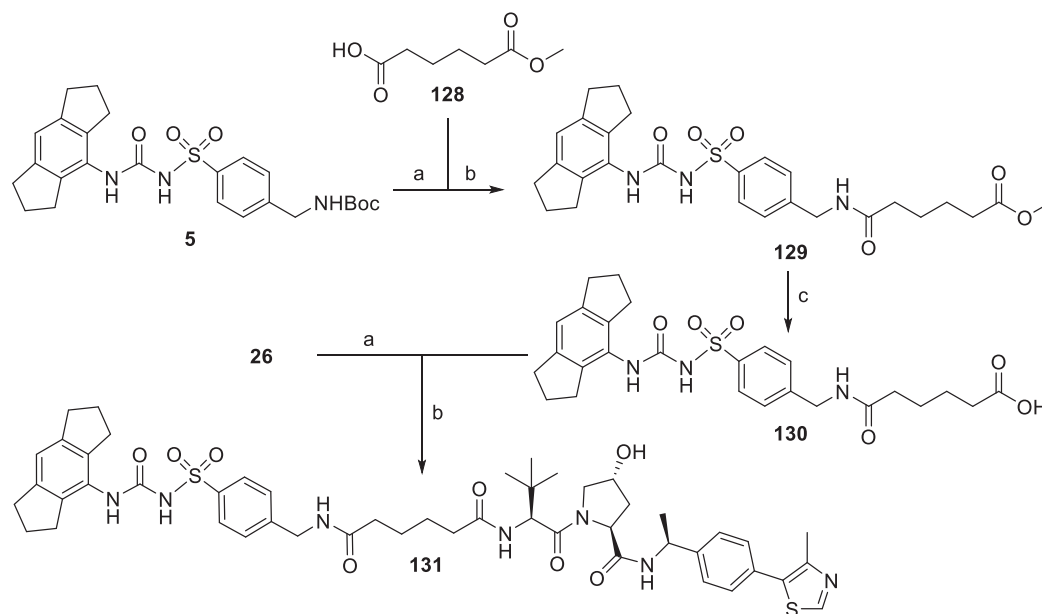
Scheme 17. Synthesis of azido to VHL or IAP PROTAC precursors.

Reagents and conditions: (a) HATU, DIPEA, DMF, rt, 2-18 h; (b) TFA, CH_2Cl_2 , rt, 2 h.

5.1.5 Final Assembly of NLRP3 PROTACs

For the final assembly of NLRP3 PROTACs, in the majority of cases NLRP3 ligands (**5**, **8**, **12**, **16**) were linked to the E3 ligase recruiting precursors as the last synthetic step. This was either achieved by HATU-promoted amide coupling reactions or CuAAC. A first set of PROTACs focussed on the application of NLRP3 ligands **5**, **8** and **12**. For this purpose, Boc-protected NLRP3 ligand **5** was deprotected with TFA and subsequently coupled with the deprotected carboxy-terminal CRBN- and VHL-addressing precursors. By this, PROTACs **120-127** were obtained (Table 1).

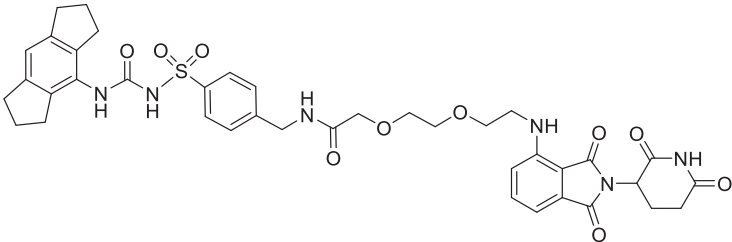
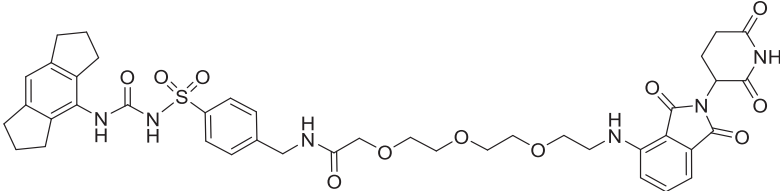
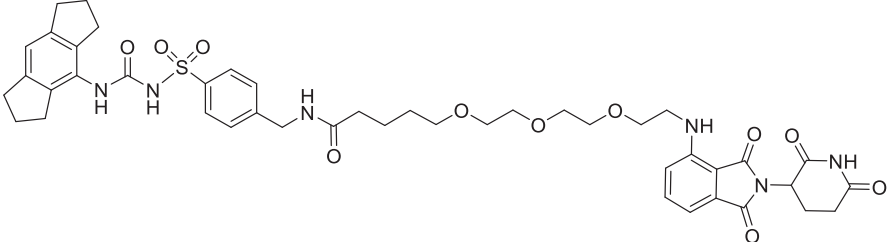
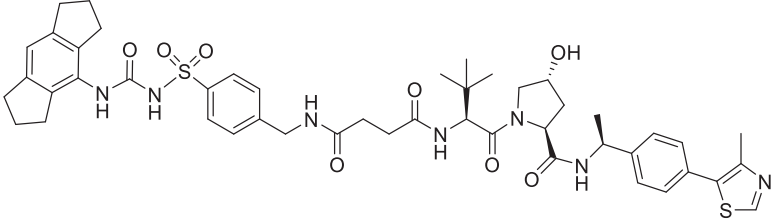
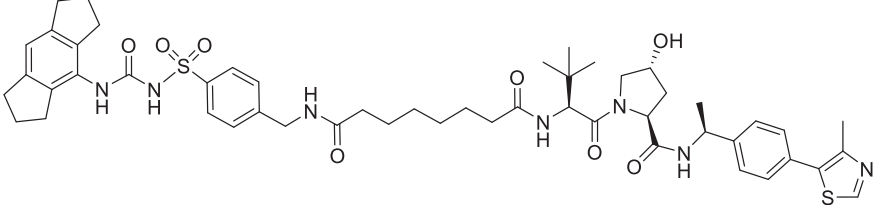
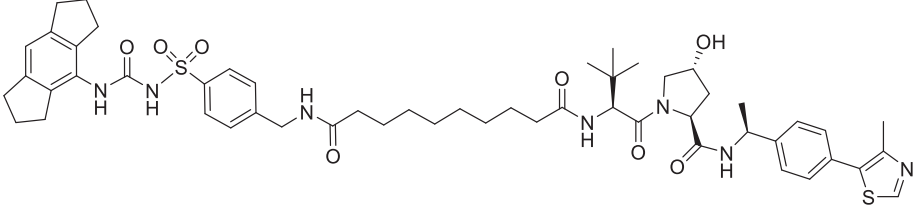
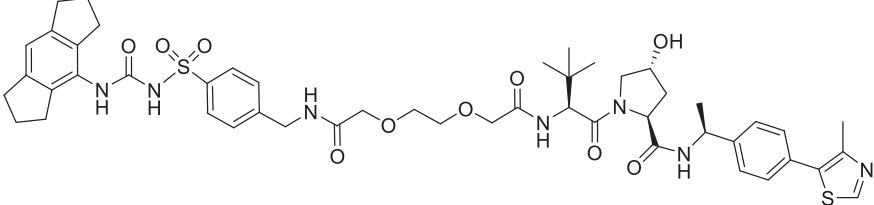
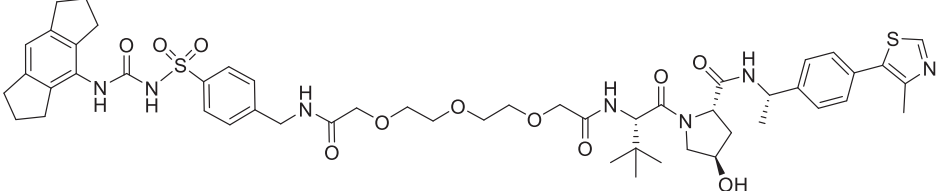
Another conceptual approach aimed at the introduction of the E3 ligase ligand as the final synthetic step. Although being unfavorable due to chemical instabilities of the NLRP3 ligand portion, one representative PROTAC was synthesized (Scheme 18). In this attempt, NLRP3 ligand **5** was deprotected and coupled with the mono-methyl ester of adipic acid (**128**). Subsequent ester cleavage of product **129** with LiOH in a mixture of H₂O, MeOH and THF afforded acid **130**. After deprotection of Boc-protected VHL ligand **26** and coupling with HATU-activated acid **130** yielded NLRP3 PROTAC **131** by means of a *vice versa* synthetic route.



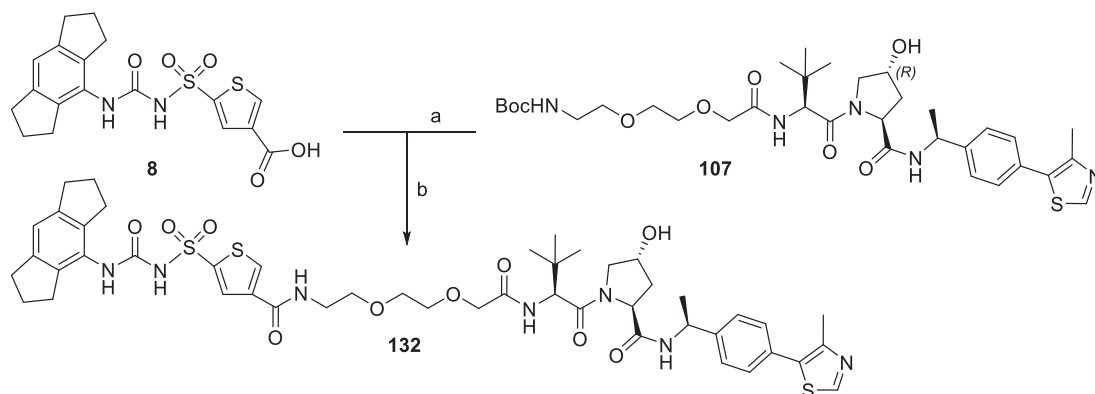
Scheme 18. Alternative synthetic route towards VHL-addressing NLRP3 PROTAC **131**.

Reagents and conditions: (a) TFA, CH₂Cl₂, rt, 2 h; (b) HATU, DIPEA, DMF, rt, 18 h; (c) LiOH, H₂O, MeOH, THF, rt, 6 h.

Table 1. Assembly of NLRP3 PROTACs *via* HATU-mediated amide coupling based on **5**.

entry	product structure	No.
5 + 87		120
5 + 88		121
5 + 119		122
5 + 98		123
5 + 100		124
5 + 102		125
5 + 103		126
5 + 105		127

In consideration of the MCC950 structure, NLRP3 ligand **8**, which is based on a five-membered heterocyclic sulfonamide, was used for PROTAC design. This was carried out by coupling **8** with amino-terminal VHL-addressing precursor **107** after its deprotection (Scheme 19). The obtained NLRP3 PROTAC **132** was the first PROTAC of the contrived set bearing a heterocyclic NLRP3 ligand.

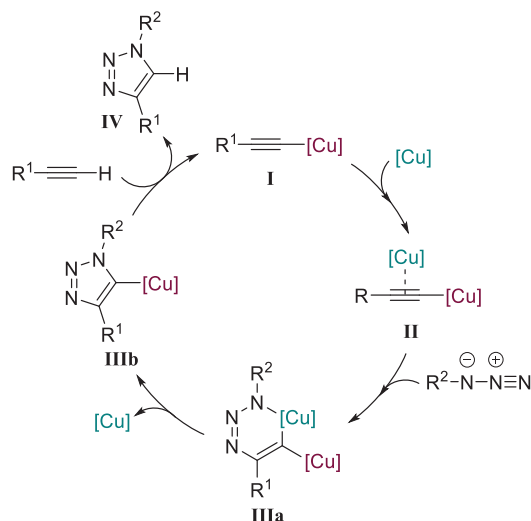


Scheme 19. Assembly of a VHL-addressing NLRP3 PROTAC with a thiophene-containing NLRP3 ligand *via* HATU-mediated amide coupling.

Reagents and conditions: (a) TFA, CH₂Cl₂, 2 h; (b) HATU, DIPEA, DMF, rt, 4 h.

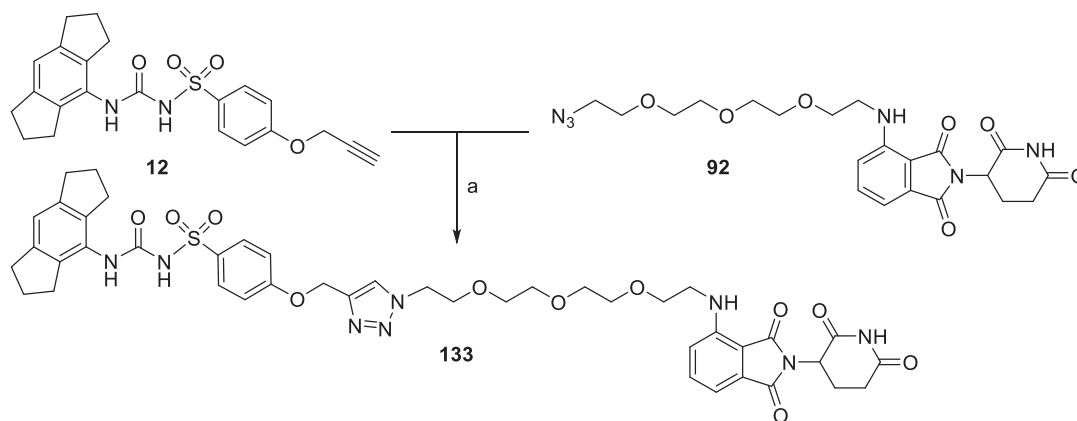
Further synthetic approaches aimed at the implementation of a ‘click’ chemistry platform for molecular chimeras targeting NLRP3. The conjugation of large molecules and in particular biomolecules takes great advantage from modular and high-yielding reactions while avoiding sensitive reaction conditions and complex purifications.²⁸² In the PROTAC field especially the CuAAC, as a catalytic version of the Huisgen 1,3-dipolar cycloaddition, is frequently applied.^{188,242,286,291–294} This reaction enables regiospecific formation of aromatic 1,4-substituted 1,2,3-triazole junctions (Scheme 20). The reaction is probably initiated by *in situ* generation of Cu(I) acetylides (**I**) and coordination of a second Cu(I) atom (**II**). After alignment of the organic azide, two carbon-nitrogen bonds are stepwise formed (**IIIa**) and copper is released (**IIIb**). Upon protonation of the obtained triazolyl-copper intermediate, 1,4-substituted triazole is formed (**IV**) and the next Cu(I) acetylide enters a catalytic cycle.^{295,296} In contrast, the thermally catalyzed 1,3-dipolar cycloaddition produces mixtures of 1,4- and 1,5-substituted triazoles. Due to their dipole moment and ability to act as hydrogen bond acceptors, 1,2,3-triazoles can be assumed as surrogates of amide junctions with high hydrolytic and chemical stability.²⁹⁷ As Cu(I) is the catalytically active oxidation level, the application of various Cu(I) sources were reported. These can be inorganic salts, coordination complexes or Cu(0), which is oxidized due to oxygen contact. Since maintaining appropriate concentrations of Cu(I) in solution remains challenging, the use of Cu(II) salts in tandem with reductants such as ascorbic

acid is favorable.^{285,297} Hence, Cu(II) sulfate and (+)-sodium L-ascorbate were utilized in catalytic amounts within this study. In order to avoid rapid oxidation of *in situ* formed Cu(I), ‘click’ reactions were performed under argon protective atmosphere.²⁹¹



Scheme 20. Hypothesized mechanism of CuAAC. Modified from Héron and Balcells.²⁹⁶

By treating alkyne-terminal NLRP3 ligand **12** with azido-terminal CRBN-addressing precursor **92** under reported conditions,²⁹¹ triazole-containing PROTAC **133** was afforded (Scheme 21). In order to avoid possible instabilities of the NLRP3 ligand during *in solution* synthesis, reaction times of the general CuAAC procedure were kept short. Following this, a tailored set of NLRP3 PROTACs (**134-151**) addressing the E3 ligases CRBN, VHL and IAP was prepared from finally optimized NLRP3 ligand **16** *via* CuAAC. In case of IAP-addressing PROTACs (**148-151**), a TFA-mediated deprotection of the Boc-protected precursors was carried out prior to ‘click’ ligation. The final set comprises 18 PROTACs with a systematic linker design concerning length and polarity (Tables 2, 3 and 4).



Scheme 21. Assembly of a CRBN-addressing NLRP3 PROTAC *via* CuAAC. Reagents and conditions: (a) (+)-sodium L-ascorbate, CuSO₄, H₂O, THF, 3 h.

Table 2. Assembly of CRBN-addressing NLRP3 PROTACs *via* CuAAC.

entry	product structure	No.
16 + 89		134
16 + 90		135
16 + 91		136
16 + 92		137
16 + 93		138
16 + 94		139
16 + 95		140

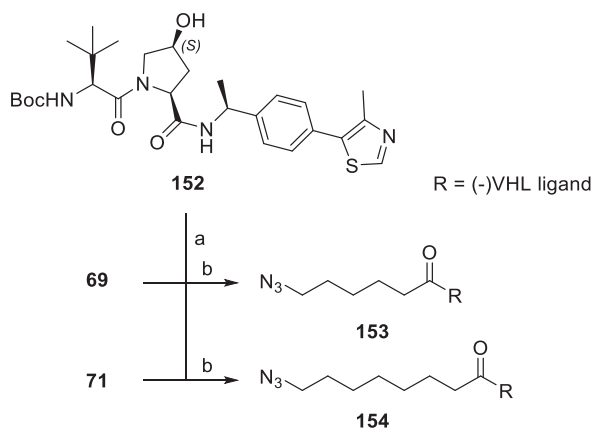
Table 3. Assembly of VHL-addressing NLRP3 PROTACs *via* CuAAC.

entry	product structure	No.
16 + 108		141
16 + 109		142
16 + 110		143
16 + 111		144
16 + 112		145
16 + 113		146
16 + 114		147

Table 4. Assembly of IAP-addressing NLRP3 PROTACs *via* CuAAC.

entry	product structure	No.
16 + 115		148
16 + 116		149
16 + 117		150
16 + 118		151

In the style of two VHL-addressing PROTACs, *i.e.* **141** and **142**, chemical negative controls were synthesized. This was achieved by installation of a (-)VHL ligand (**152**), bearing an inverted stereocenter at the proline moiety (Scheme 22). The two negative control compounds **155** and **156** were obtained by coupling the precursors **153** and **154** with NLRP3 ligand **16** *via* CuAAC (Table 5).



Scheme 22. Design of precursors with an inverted stereocenter for chemical negative controls.

Reagents and conditions: (a) TFA, CH₂Cl₂, rt, 2 h; (b) HATU, DIPEA, DMF, rt, 18 h.

Table 5. Assembly of chemical negative controls *via* CuAAC.

entry	product structure	No.
16 + 153		155
16 + 154		156

5.1.6 Physicochemical and Biophysical Evaluation of PROTACs

As already outlined, PROTACs usually neglect Lipinski's rule of five.⁷ This is due to their enormous molecular weight (MW), increased topological polar surface area (TPSA) and the high numbers of rotatable bonds, hydrogen bond donors and acceptors. Although it was noted that these rules are not obligatorily suitable for PROTACs, because of disregarding molecules' occupied three-dimensional structure in changing environments,²⁹⁸ initial descriptors of drug-likeness were obtained chromatographically. The HPLC measurements were carried out by Dr. Christian Steinebach (Pharmaceutical Institute, University of Bonn, Bonn). One of them is the experimental distribution coefficient at a physiological pH of 7.4 ($\log D_{7.4}$) as a measure for lipophilicity. The experimentally determined percentage of PPB represents a first pharmacokinetic descriptor (Table 6). From these data, it is apparent that alkyl linkers as well as longer linkers irrespective of their constitution exhibit increasing $\log D_{7.4}$ values, corresponding with higher lipophilicity.

In the course of the NLRP3 PROTAC library syntheses, valuable experiences regarding structure-stability relationships of the POI ligands were acquired. Hence, the biophysical characterization of PROTACs was focused on the latest subset comprising compounds **134-151**. The selected compounds were subjected to a thermal stability assay as an indicator for protein stability. It is well recognized that proteins upon ligand binding can be protected from unfolding through thermal stress. Assessment of proteins' thermal stability hence serves as a measure for binary complex formation between a protein and a ligand.²⁹⁹ For this purpose, PROTACs **134-147** were evaluated in a nano differential scanning fluorimetry (nanoDSF) experiment. This methodology measures the change of the intrinsic fluorescence of tryptophan residues of the envisaged protein as a result of protein unfolding at increasing temperatures. By this, the melting temperatures of proteins (T_m) and the difference in melting temperatures (ΔT_m) after ligand treatment are determined.²⁹⁹ All the nanoDSF measurements reported in this chapter and for that purpose required recombinant proteins were performed and prepared by Dominic Ferber, Maximilian Schmitz and Dr. Michael Marleaux under supervision of Prof. Dr. Matthias Geyer (Institute of Structural Biology, University of Bonn, Bonn, Germany). Thermal stability of NLRP3 in presence of PROTACs **134-147** was investigated as well as in the presence of MCC950 as a positive control. Human NLRP3 was used as maltose-binding protein (MBP)-fusion protein (MBP-NLRP3). All investigated PROTACs increased the melting temperature of NLRP3 in a comparable order of magnitude as MCC950 (ca. 3-4 °C). The E3 ligase ligands, *i.e.* pomalidomide and VH298, just slightly increased the melting temperatures (<1 °C) compared to the DMSO control (Figures 18 and 19).

Table 6. Physicochemical and pharmacokinetic properties of NLRP3 PROTACs.

No.	NLRP3 ligand	E3 ligase	MW (g/mol) ^a	linker		<i>elogD</i> _{7.4} ^b	PPB (%) ^c
				atoms	type		
120	5	CRBN	786.85	8	PEG	1.9	97
121	5	CRBN	830.90	11	PEG	1.9	96
122	5	CRBN	872.98	14	Mixed	2.0	96
123	5	VHL	912.13	4	Alkyl	2.0	97
124	5	VHL	968.24	8	Alkyl	2.3	97
125	5	VHL	996.29	10	Alkyl	2.5	97
126	5	VHL	972.18	8	PEG	2.1	n.d.
127	5	VHL	1016.24	11	PEG	2.1	96
131	5	VHL	940.18	6	Alkyl	2.1	n.d.
132	8	VHL	978.21	9	PEG	2.1	97
133	12	CRBN	884.96	11	PEG	2.4	97
134	16	CRBN	828.96	6	Alkyl	2.5	97
135	16	CRBN	857.01	8	Alkyl	3.0	97
136	16	CRBN	860.96	8	PEG	2.1	97
137	16	CRBN	905.01	11	PEG	2.1	96
138	16	CRBN	949.06	14	PEG	2.2	96
139	16	CRBN	993.12	17	PEG	2.2	96
140	16	CRBN	1181.38	30	Mixed	2.6	95
141	16	VHL	1014.29	6	Alkyl	2.3	97
142	16	VHL	1042.34	8	Alkyl	2.5	97
143	16	VHL	1046.29	8	PEG	2.2	96
144	16	VHL	1090.34	11	PEG	2.2	96
145	16	VHL	1134.39	14	PEG	2.3	96
146	16	VHL	1178.45	17	PEG	2.3	96
147	16	VHL	1366.71	30	Mixed	2.7	95
148	16	IAP	1053.35	6	Alkyl	n.d.	n.d.
149	16	IAP	1129.40	11	PEG	2.3	n.d.
150	16	IAP	1173.46	14	PEG	2.3	n.d.
151	16	IAP	1217.51	17	PEG	2.4	n.d.

^a Molecular weight; ^b Experimental distribution coefficient at pH 7.4; ^c Experimentally determined percentage of compound bound to human serum albumin; n.d. not determined.

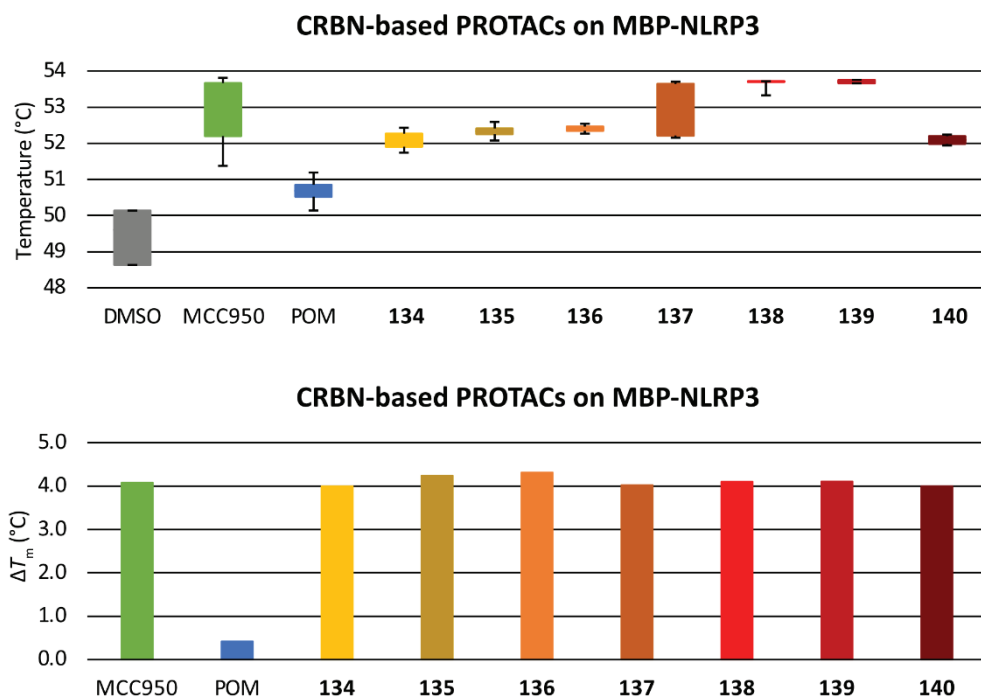


Figure 18. Thermal stability assay of MBP-NLRP3 treated with CRBN-based PROTACs. nanoDSF measurements of 10 μ M MBP-NLRP3 treated with 2% DMSO or 30 μ M of one of the indicated test compounds (MCC950, pomalidomide, or PROTACs **134-140**). Melting temperatures (T_m) of MBP-NLRP3 from at least two independent measurements are depicted as boxplots (top). Changes in melting temperatures (ΔT_m) of MBP-NLRP3 are depicted as a bar chart (bottom).

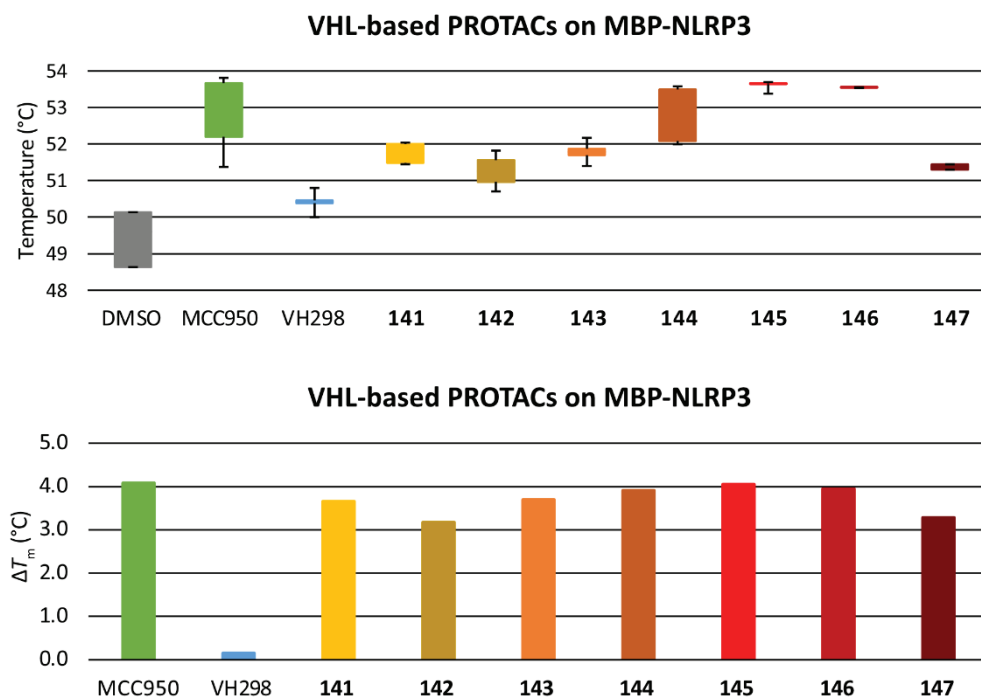


Figure 19. Thermal stability assay of MBP-NLRP3 treated with VHL-based PROTACs. nanoDSF measurements of 10 μ M MBP-NLRP3 treated with 2% DMSO or 30 μ M of one of the indicated test compounds (MCC950, VH298, or PROTACs **141-147**). Melting temperatures (T_m) of MBP-NLRP3 from at least two independent measurements are depicted as boxplots (top). Changes in melting temperatures (ΔT_m) of MBP-NLRP3 are depicted as a bar chart (bottom).

In another experimental setting, PROTACs were evaluated regarding their ability to stabilize the addressed E3 ligases CRBN and VHL. A recombinant complex of CRBN with a modified version of the adaptor protein DDB1 Δ B (CRBN/DDB1 Δ B) was used for the evaluation of CRBN-addressing PROTACs **134-140**. MCC950 did not increase the melting temperature of CRBN/DDB1 Δ B, compared to the DMSO control. Pomalidomide and compounds **134-140** instead caused a clear rise in melting temperatures (Figure 20). Hence, the synthesized CRBN-addressing PROTACs **134-140** are able to bind both the POI and the E3 ligase.

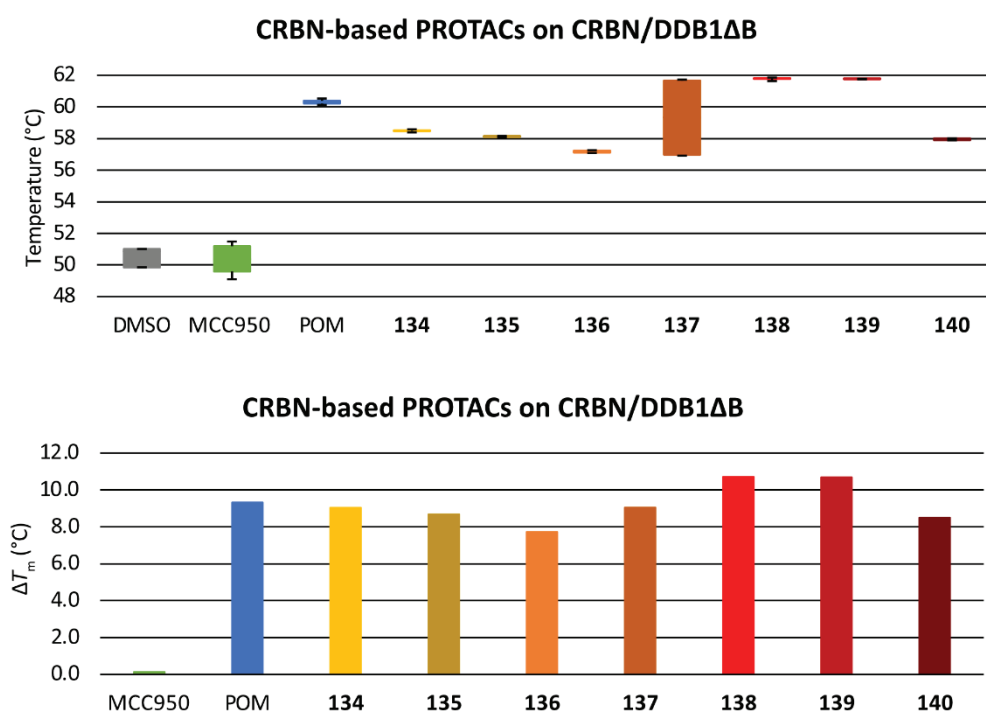


Figure 20. Thermal stability assay of CRBN/DDB1 Δ B treated with CRBN-addressing PROTACs. nanoDSF measurements of 10 μ M CRBN/DDB1 Δ B treated with 2% DMSO or 30 μ M of one of the indicated test compounds (MCC950, pomalidomide, or PROTACs **134-140**). Melting temperatures (T_m) of CRBN/DDB1 Δ B from at least two independent measurements are depicted as boxplots (top). Changes in melting temperatures (ΔT_m) of CRBN/DDB1 Δ B are depicted as a bar chart (bottom).

The affinities of VHL-addressing PROTACs **141-147** to the VHL protein were determined in an analog manner using VHL in complex with the adaptor proteins elongin B and C (VBC). Again, the NLRP3 ligand MCC950 did not show stabilizing effects on VBC when compared to the DMSO control. In contrast, the VHL ligand VH298 as well as PROTACs **141-147** had a considerable stabilizing effect on VBC deduced from changes in melting temperature higher than 10 $^{\circ}$ C (Figure 21). These findings prove PROTACs **141-147** to be efficient chimeras binding NLRP3 and VHL in binary complexes.

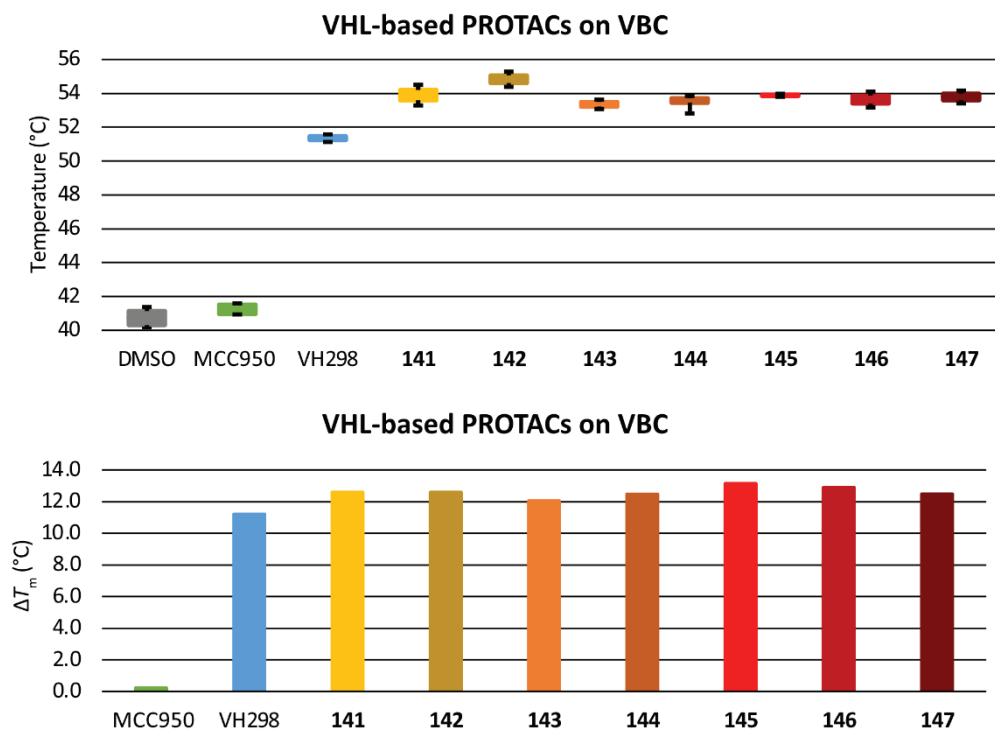
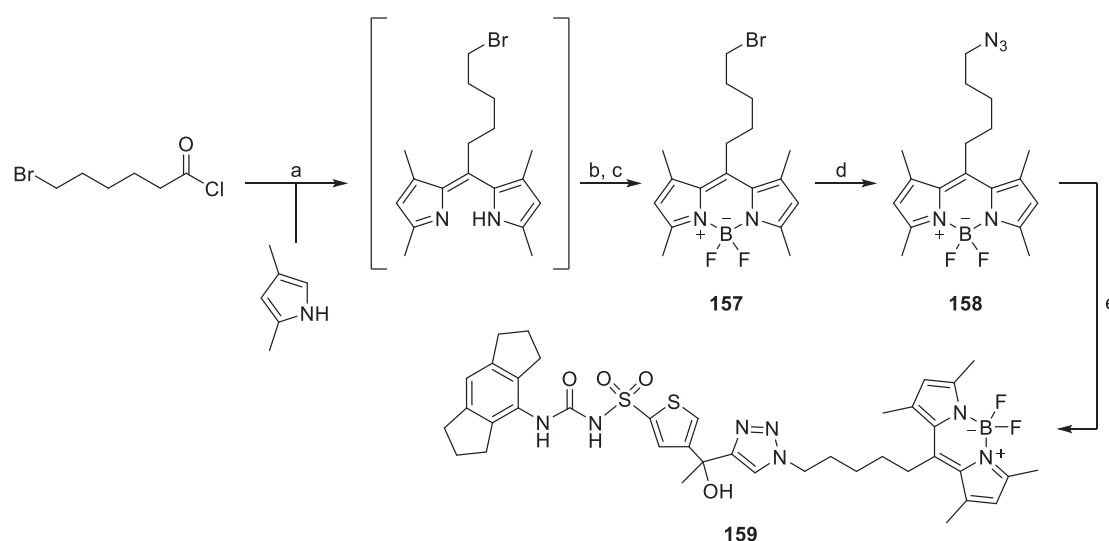


Figure 21. Thermal stability assay of VBC treated with VHL-addressing PROTACs. nanoDSF measurements of 10 μ M VBC treated with 2% DMSO or 30 μ M of one of the indicated test compounds (MCC950, VH298, or PROTACs **141-147**). Melting temperatures (T_m) of VBC from at least two independent measurements are depicted as boxplots (top). Changes in melting temperatures (ΔT_m) of VBC are depicted as a bar chart (bottom).

5.2 Design of a NLRP3 Probe with a BODIPY Reporter

As an expansion of the fluorescent NLRP3 probe set, a refined compound was prepared. Since the previous fluorescent probes based on a coumarin 343 fluorophore,³⁰⁰ an alternative boron-dipyrromethene (BODIPY) reporter unit was herein chosen as luminophore. BODIPYs are commonly applied dyes for biological labeling approaches due to their thermal and chemical robustness, excellent spectroscopic properties and high adaptability. Their observed distinct quantum yields are deduced from an extraordinary delocalization of π -electrons in resemblance to cyanines. The spectroscopic properties of BODIPYs can be adjusted by introducing substituents to the pyrroles.³⁰¹ Herein, electrophilic 6-bromohexanoyl chloride was employed to bridge two molecules of 2,4-dimethyl pyrrole (Scheme 23). The obtained *in situ* intermediate was reacted with boron trifluoride diethyl etherate in the presence of TEA to form the fluorine BODIPY **157**. By this, a bromoalkane handle was installed at the 8-position, also referred to as *meso* site. Subsequent azidolysis with NaN₃ in DMF yielded **158**, as previously reported.³⁰² The following CuAAC with NLRP3 ligand **16** afforded the BODIPY-equipped NLRP3 probe **159**.

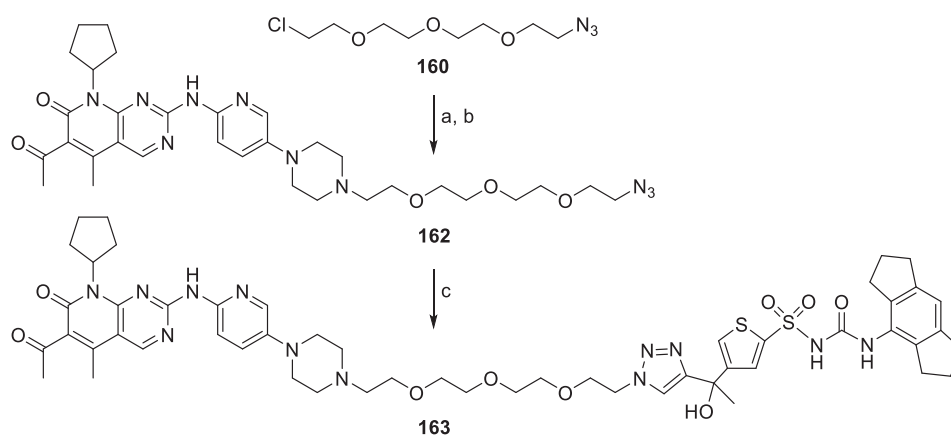


Scheme 23. Design of a fluorescent NLRP3 probe employing a BODIPY reporter unit.

Reagents and conditions: (a) CH_2Cl_2 , 0 °C for 30 min, rt for 30 min; (b) TEA, 0 °C, rt, 10 min; (c) $\text{BF}_3 \cdot \text{OEt}_2$, rt, 14 h; (d) NaN_3 , DMF, 50 °C, 18 h; (e) **16**, (+)-sodium L-ascorbate, CuSO_4 , H_2O , THF, 3 h.

5.3 Initial Attempts in Addressing Zswim2

Researchers in the field of TPD are perpetually searching for new E3 ligases being viable for PROTAC technology or molecular glues. Beside the herein addressed E3 ligases CRBN, VHL and IAP further have been identified to be suitable for a PROTAC approach. A crucial step towards establishment of a new E3 ligase for PROTAC application is the development of appropriate ligands.²⁵⁰ As apparent from an off-target screening assay, E3 ligase Zswim2 (zinc finger SWIM domain-containing protein 2)^{303,304} was found to interact with the photoaffinity probe IMP2070 (Figure 8), a MCC950 relative.¹⁰⁷ Hence, an initial attempt aimed at the design of a PROTAC addressing Zswim2. This was achieved by linking the NLRP3 ligand **16** to palbociclib, a ligand for the established TPD substrate cyclin-dependent kinase 6 (CDK6).^{211,242,305} CDK6 is a crucial player in cell cycle progression and hence represents an important pharmacological target for cancer treatment.³⁰⁶ In this scenario, the NLRP3 ligand served as a ligand for Zswim2. For this purpose, linker **160** was obtained through one-sided azidolysis.³⁰⁷ Conversion of **160** under Finkelstein conditions and subsequent reaction of the intermediate alkyl iodide with palbociclib (**161**) yielded **162**.²¹¹ A CuAAC with **16** afforded the heterobifunctional molecule **163** (Scheme 24).



Scheme 24. Synthesis of a CDK6-targeting chimeric molecule for addressing Zswim2.

Reagents and conditions: (a) NaI, acetone, 60 °C, 48 h; (b) palbociclib (**161**), DIPEA, DMF, DMSO, 80 °C, 48 h; (c) **16**, (+)-sodium L-ascorbate, CuSO₄, H₂O, THF, 3 h.

5.4 Conclusions

The development of chimeric molecules described in this chapter pursues different aims. The identification of potential degraders of the inflammatory driver NLRP3 started with the selection of a POI ligand scaffold and its adaption for PROTAC approaches. MCC950 as an earlier clinical candidate and well-known pharmacological tool compound exhibited promising properties: (i) reversible non-covalent binding to the NACHT domain of NLRP3, which is a prerequisite for the catalytic mode of action of PROTACs, (ii) availability of structure-activity relationships allowing for a suggestion of possible linker attachment points, (iii) synthetic accessibility for structural diversity. Together with the generation of a large set of precursor molecules under the consideration of the three most-well established E3 ligases in the PROTAC field, a panel of 29 NLRP3 PROTACs was designed and synthesized. Among these, 18 were initially evaluated in terms of drug-likeness and biophysical properties.

The development of the clickable NLRP3 ligand **16**, on the basis of a high affinity binding thiophene analog of MCC950,³⁰⁸ opened up new opportunities in the design and generation of chimeric multipurpose NLRP3 ligands. A BODIPY-based fluorescent probe was prepared from scratch as an expansion of the reported probe portfolio in order to be applied in microscopy and multiple inflammasome assays.

A completely new approach was followed by the synthesis of a heterobifunctional molecule consisting of the CDK6 ligand palbociclib and **16**. Inspired by the proteomics data, obtained from a photoaffinity probe for MCC950,¹⁰⁷ led to the idea of a CDK6 targeting PROTAC addressing the E3 ligase Zswim2. The conceptualized compound allows for preliminary investigations and may shed light on the rather unknown ligase.

6 Summary and Perspective

The restoration of balanced protein function is a fundamental and complex process, implicated in the treatment of myriad diseases and disorders. More than 20 years ago, a novel mechanistic concept coined an innovative drug modality.¹⁰ Since then, enormous drug development efforts have been devoted to the design and implementation of PROTACs catalyzing the proteolytic degradation of harmful proteins. Beside the exploration of the extended chemical space, it is reasonable to also focus on established or latest identified target proteins in order to make them accessible for a TPD approach.

A major key player in innate immunity is NLRP3. A growing body of research in both, academia and industry, aims at the development of inhibitors. Within this work particular attention was paid to the complementary attempt to degrade NLRP3 by hijacking the UPS *via* PROTACs. In the beginning, a robust protocol to transform aromatic amines into their corresponding isocyanates was applied. Together with the syntheses of diversely functionalized sulfonamides, a panel of sulfonylureas was prepared. A selection of them, regarding substituent effects, was characterized considering the chemical stability at different *pH* values. While a first set of NLRP3 PROTACs was prepared at an earlier experimental stage, lessons learned from the structure-stability relationships guided the design of the second generation NLRP3 PROTACs. This subset includes compounds **134-151** and was exclusively prepared *via* a final CuAAC employing POI ligand **16** and a tailored series of clickable azido-terminal precursor molecules. With this second series in hand, first physicochemical and pharmacokinetic parameters were determined. Next, biophysical evaluation was carried out by means of a thermal stability assay. All investigated PROTACs (**134-151**) exhibited adequate stabilization of NLRP3, indicating reasonable affinity. The CRBN-addressing (**134-140**) and VHL-addressing (**141-147**) PROTACs were further proven to successfully stabilize their respective ligase complexes, *i.e.* CRBN/DDB1 Δ B and VBC.

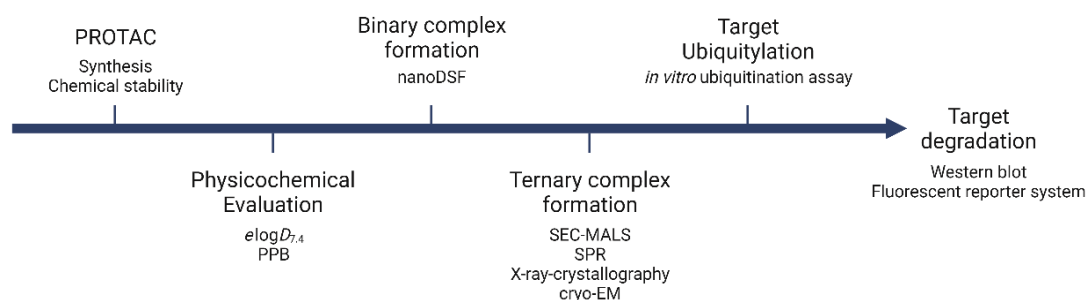


Figure 22. Possible route towards NLRP3 PROTACs. Modified from Hughes and Ciulli.³⁰⁹

The following steps (Figure 22) include investigation of ternary complex formation of POI, PROTAC and E3 ligase, *e.g.* by size-exclusion chromatography coupled to a multiangle-light scattering detector (SEC-MALS) or by SPR. Subsequent ubiquitination of the POI will be studied in an *in vitro* ubiquitination assay. Lastly, protein levels of the target protein will be examined as a measure for degradation. In principle, this can be achieved with different methodologies, *e.g.* by Western blot analysis or by harnessing a fluorescent reporter system.³¹⁰ In order to provide evidence for the proposed mode of action of NLRP3 PROTACs, various control experiments will be performed. These comprise the application of the chemical negative controls **155** and **156**, the implementation of competition experiments with both POI and E3 ligase ligands, and eventually the inhibition of the UPS with proteasome inhibitors such as MG132. Once a hit compound is identified, NLRP3 protein could be purified in the presence of the PROTAC followed by the addition of the addressed E3 ligase in order to obtain crystals of the ternary complex. Hence, the occupied three-dimensional PROTAC structure might be deducible from X-ray crystallography or cryo-EM experiments. Following this, especially the linker structure could be optimized in terms of increased rigidity, ultimately locking the PROTAC molecule in its three-dimensional structure.

Another project aimed at the design of fluorescent and biotin probes as tool compounds for biochemical experiments on the NLRP3 inflammasome. Two fluorescent probes, employing a coumarin reporter, were found to be NLRP3 inhibitors while one of them was successful to mark NLRP3 in cell as observed *via* confocal microscopy. Based on the improved clickable NLRP3 ligand **16**, a further fluorescent probe was prepared, being equipped with a BODIPY fluorophore. In the next steps, probe **159** can be evaluated regarding the inhibitory potency of IL-1 β release from various cell lines. Furthermore, it might be used in fluorescence polarization displacement assays in order to evaluate new inhibitor candidates, occupying the same binding site. This approach was in principal already pursued using a comparable BODIPY probe (Figure 8).¹⁰² An interesting attempt might be the design of a smaller probe, consisting of a BODIPY scaffold replacing the 1,2,3,5,6,7-hexahydro-*s*-indacene in MCC950 (Figure 23).

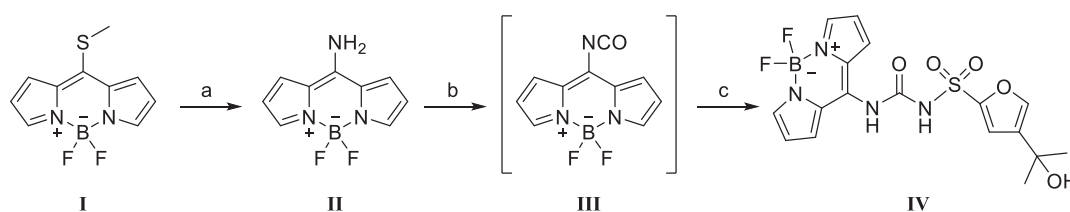


Figure 23. Possible route towards fluorine BODIPY probe IV.

The starting reagent 8-methylthio-BODIPY (**I**) can be obtained from pyrrole in a reported three-step procedure.³¹¹ The thiomethyl group is introduced by the sequential use of thiophosgene and iodomethane. The BODIPY scaffold is formed by treating the intermediate with boron trifluoride diethyl etherate. Subsequent reaction of **I** with an ammonia source, *e.g.* ammonium hydroxide or ammonia in dioxane, in an appropriate solvent is reported to give amine **II**.^{312,313} Isocyanation of **II** under triphosgene treatment could deliver intermediate **III**, which in turn can be reacted with the corresponding deprotonated sulfonamide to give probe **IV**. The newly generated probe would be interesting concerning both, the spectroscopic properties of the fluorine BODIPY reporter and its inhibitory potency regarding the NLRP3 inflammasome.

An additional approach was directed at the initial characterization of MCC950-like diaryl sulfonylureas as ligands for the rather unknown E3 ligase Zswim2. Heterobifunctional probe **163** was generated utilizing the CDK6 inhibitor palbociclib and the proper NLRP3 ligand **16**. Hence, it is yet to be investigated whether **163** is able to address Zswim2. Determining the protein levels of CDK6 in different cell lines *via* Western blot upon PROTAC treatment could serve as an indicator for target engagement. The selection of cell lines should include those from mouse and human testicular tissue, due to the reported abundance of Zswim2.^{303,304} To provide further evidence, it would be reasonable to develop additional tool compounds. A homobifunctional probe constituted of two molecules of clickable ligand **16** bridged by a diazido linker would afford probe **V** (Figure 24). In the case of a Zswim2 involvement and ternary complex formation, the degradation of Zswim2 itself could be measured by means of Western blot analysis.

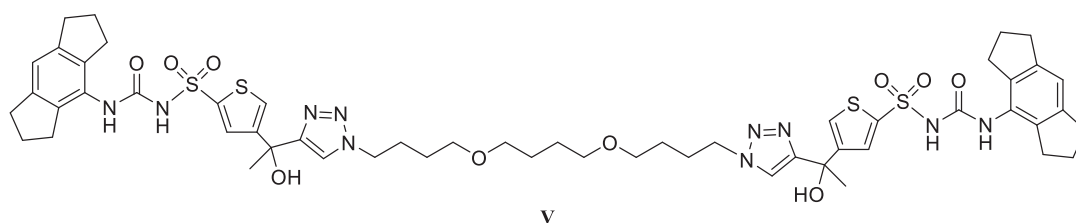


Figure 24. Prototypical homobifunctional probe **V**.

The last project aimed at the development of the first selective non-hydroxamate PROTACs targeting HDAC6. By introducing a DFMO warhead as innovative ZBG, six PROTACs addressing either the CRBN or VHL E3 ligase were generated. Each of them demonstrated selective inhibition of HDAC6's catalytic activity and significant potency in degrading HDAC6 in MM.1S multiple myeloma cells. Apart from that, two selected compounds were mechanistically evaluated in more detail. It was proven that HDAC6 was degraded selectively,

since protein levels of HDAC1 and HDAC4 remained unaffected. The mode of action was proven to be UPS-dependent, as treatment with the neddylation inhibitor MLN4924 protected HDAC6 from proteolytic degradation. Furthermore, the use of competing ligands at the respective proteins, *i.e.* HDAC6, CRBN and VHL, successfully abolished PROTAC-dependent protein degradation. Tailored chemical negative controls in terms of modified E3 ligase ligands were also incapable in inducing HDAC6 degradation. Since then, the underlying mechanism of this novel DFMO-based ZBG regarding its potency to selectively inhibit HDAC6 was elucidated in detail, hypothesizing a long-lived enzyme-inhibitor complex due to a two-step slow-binding profile.^{314–316} These mechanistic studies pave the way for the design of future excellently selective HDAC6 inhibitors and PROTACs. In addition, HDAC6-targeting PROTACs are also interesting concerning their capability in inhibiting the NLRP3 inflammasome. Recent reports attributed an IL-1 β release inhibitory potency to hydroxamic acid-based HDAC6 PROTACs.^{198,201} Since this correlation may rather depend on the abrogated catalytic activity of HDAC6, this should also be valid for DFMO-based inhibitors as well as PROTACs. Nevertheless, such studies have to be performed to provide further evidence.

7 Experimental Section

7.1 General Methods and Materials

Commercially available chemicals and starting reagents were purchased from abcr, Acros Organics, BLDpharm, Carl Roth, Fisher Scientific, Fluorochem, Sigma Aldrich/Merck, Tokyo Chemical Industry (TCI) or VWR Chemicals and were used without further purification. All reactions were conducted using anhydrous solvents. Thin-layer chromatography (TLC) was carried out on Merck silica gel (60 F₂₅₄) aluminum sheets. Detection was performed with UV light at 254 and 360 nm or with AgNO₃ (10% m/v in H₂O) or ninhydrin (0.2% m/v in EtOH) staining. Spot identification was supported by TLC-MS on an Advion Plate Express plate reader (Ithaca, United States of America) coupled with an Advion expression L compact mass spectrometer with APCI source. Acros Organics silica gel (0.060-0.200 mm, 60 Å) was used for preparative column chromatography. Preparative flash column chromatography was performed on a puriFlash XS520Plus (Interchim, Montluçon, France) with diode-array detection (DAD) from 200 to 400 nm. Uncorrected melting points (mp) were determined on a Büchi (Essen, Germany) 510 or a Büchi M-560 melting point apparatus.

NMR spectra (¹H, ¹³C) were recorded on a Bruker Avance DRX 500 or on a Bruker Avance III 600 spectrometer. Spectra were processed and analyzed in MestReNova. Chemical shifts (δ) are given in parts per million (ppm) and coupling constants (J) are given in hertz (Hz). Spin multiplicities are given as s (singlet), d (doublet), t (triplet), q (quartet), quint (quintet), m (multiplet) or dd (doublet of doublets). Multiplicities are reported as they were measured and might disagree with the expected multiplicity of a signal. LC-ESI-MS analyses were carried out on an API 2000 mass spectrometer (AB Sciex, Darmstadt, Germany) coupled with an Agilent HPLC HP 1100 (Santa Clara, United States of America) using an EC50/2 Nucleodur C18 Gravity 3 μ m column (Macherey-Nagel, Düren, Germany) or on an Agilent Infinity Lab LC/MSD-system (Santa Clara, United States of America) coupled with an Agilent HPLC 1260 Infinity II using an EC50/2 Nucleodur C18 Gravity 3 μ m column (Macherey-Nagel, Düren, Germany). Samples (1 mg/mL) were dissolved in MeOH, H₂O (each may contain 2 mM NH₄OAc) or MeCN. A volume of either 8 μ L or 2 μ L were injected into the tempered column at 25 °C or 40 °C, respectively. The flow rate was either 0.3 mL/min or 0.5 mL/min. For LC-MS analyses, the following gradients were applied: (i) 90% H₂O + 2 mM NH₄OAc to 100% MeOH + 2 mM NH₄OAc, then 100% MeOH + 2 mM NH₄OAc to 15 min or 20 min; (ii) 90% H₂O + 2 mM NH₄OAc to 100% MeCN, then 100% MeCN to 15 min or 20 min. Acidic

modifiers (HCOOH, CH₃COOH) were added to the mobile phase if stated. The purity of synthesized compounds was determined by LC-DAD. HR-ESI-MS spectra were recorded on a Bruker micrOTOF-Q mass spectrometer coupled with a HPLC Dionex UltiMate 3000 (Thermo Scientific, Braunschweig, Germany) or on a LTQ Orbitrap XL (Thermo Fisher Scientific, Bremen, Germany).

7.2 *elogD*_{7.4} Measurements

The determination of the *elogD*_{7.4} values was performed by a chromatographic method similar to previous reports,^{211,212} using a Jasco 2000 Series HPLC. The system was calibrated by plotting the retention times of six different reference substances (atenolol, metoprolol, labetalol, diltiazem, triphenylene, permethrin) *versus* their known distribution coefficient values at pH 7.4 from literature^{212,317} to obtain a calibration line ($R^2 \geq 0.98$). Subsequently, the mean retention times ($n = 2$) of the analytes were taken to calculate their *elogD*_{7.4} values with aid of the calibration line.

These experiments were performed by Dr. Christian Steinebach, Pharmaceutical Institute, University of Bonn, Bonn, Germany.

7.3 Plasma Protein Binding Studies

Plasma protein binding was estimated by correlating the logarithmic retention times of the analytes on a CHIRALPAK HSA 50 × 3 mm, 5 μm column with the literature known PPB values (converted into logK values) of ten drugs (warfarin, ketoprofen, budesonide, nizatidine, indomethacin, acetylsalicylic acid, carbamazepine, piroxicam, nicardipine, cimetidine).²¹³ Samples were dissolved in MeCN/DMSO 9:1 to achieve a final concentration of 0.5 mg/mL. The mobile phase A was 50 mM ammonium acetate adjusted to pH 7.4 with aqueous ammonia, while mobile phase B was *i*PrOH. The flow rate was set to 1.0 mL/min, the UV detector was set to 254 nm, and the column temperature was kept at 30 °C. After injecting 2 or 3 μL of the sample, a linear gradient from 100% A to 30% *i*PrOH in 5.4 min was applied. From 5.4 to 18 min, 30% *i*PrOH was kept, followed by switching back to 100% A in 1.0 min and a re-equilibration time of 6 min. With the aid of the calibration line ($R^2 = 0.96$), the logK values of new substances were calculated and converted to their PPB values. At least two independent measurements of each analyte were performed.

These experiments were performed by Dr. Christian Steinebach, Pharmaceutical Institute, University of Bonn, Bonn, Germany.

7.4 Thermal Stability Measurements

Thermal stability measurements were performed by nano-differential scanning fluorimetry (nano-DSF) using a Prometheus NT.48 device (NanoTemper Technologies, Munich, Germany). The excitation power was set to 15-20%, depending on the analyzed protein. Thermal stability was monitored from 20 to 90 °C at a heating rate of 1.5 °C/min. Each sample contained 10 µM of the respective protein (MBP-NLRP3 (provided by Michael Marleaux, Institute of Structural Biology, University of Bonn, Bonn, Germany), VBC, CRBN/DDB1ΔB (both provided by Maximilian Schmitz, Institute of Structural Biology, University of Bonn, Bonn, Germany) and 30 µM of the respective PROTAC (**134-147**) or control compound (VH298, CRID3) (stock solution: 1.5 mM in DMSO), the total volume of each sample was 30 µL. The negative controls contained 2% DMSO instead of a compound.

These experiments were performed by Dominic Ferber, Institute of Structural Biology, University of Bonn, Bonn, Germany.

7.5 Illustrations

Figures 1, 3, 4, 5, 6, 10 and 22 were created using BioRender (<http://www.biorender.com>).

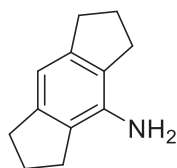
7.6 Preparation of Compounds

7.6.1 General Procedure for CuAAC

The NLRP3 ligand (**16**, if not stated otherwise) (1 eq), the corresponding azido-precursor (1 eq), (+)-sodium L-ascorbate (0.2 eq) and copper(II) sulfate pentahydrate (0.2 eq) were dissolved in a mixture of THF (2 mL) and water (2 mL). The reaction mixture was stirred for 3 h at room temperature under argon. The mixture was concentrated under high vacuum and purified by flash column chromatography.

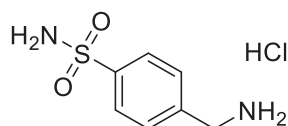
7.7 Compounds

1,2,3,5,6,7-Hexahydro-*s*-indacen-4-amine (**1**)^{107,219,318}



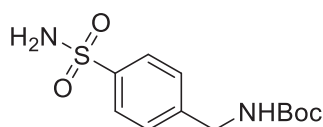
Compound **1** was purchased from abcr (Karlsruhe, Germany). However, it was also prepared as described before.³⁰⁸

4-(Aminomethyl)benzenesulfonamide hydrochloride (**3**)



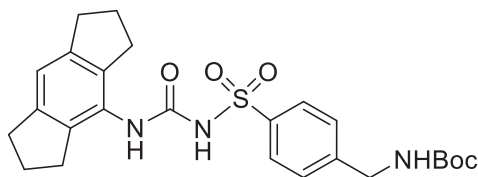
Compound **3** was purchased from abcr (Karlsruhe, Germany).

tert-Butyl (4-sulfamoylbenzyl)carbamate (**4**)³¹⁹



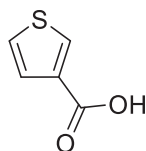
Compound **4** was prepared as described before.³⁰⁰

Yield 89%; mp: 144-146 °C; ¹H NMR (600 MHz, DMSO-*d*₆) δ 1.39 (s, 9H, C(CH₃)₃), 4.18 (d, *J* = 6.1 Hz, 2H, CH₂), 7.28 (s, 2H, SO₂NH₂), 7.40 (d, *J* = 8.2 Hz, 2H, CH_{arom.}), 7.46 (t, *J* = 5.9 Hz, 1H, CONH), 7.76 (d, *J* = 8.2 Hz, 2H, CH_{arom.}); ¹³C NMR (151 MHz, DMSO-*d*₆) δ 28.2 (C(CH₃)₃), 43.0 (CH₂), 78.0 (C(CH₃)₃), 125.6, 127.2, 142.5, 144.2 (C_{arom.}), 155.8 (CONH); LC-MS (ESI) (90% H₂O to 100% MeOH in 10 min, then 100% MeOH to 20 min, DAD 220-400 nm), *t*_R = 8.45 min, 100% purity, *m/z* [M + H]⁺ calcd for C₁₂H₁₈N₂O₄S 287.11, found 287.0.

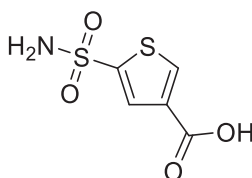
tert-Butyl**(4-(N-((1,2,3,5,6,7-hexahydro-s-indacen-4-yl)carbamoyl)sulfamoyl)benzyl)carbamate (5)**

Compound **5** was prepared as described before.³⁰⁰

Yield 48%; mp: >250 °C (decomposition); ¹H NMR (500 MHz, DMSO-*d*₆) δ 1.39 (s, 9H, C(CH₃)₃), 1.92 (quint, *J* = 7.4 Hz, 4H, CH₂ indacene), 2.53 (t, *J* = 7.4 Hz, 4H, CH₂ indacene), 2.77 (t, *J* = 7.4 Hz, 4H, CH₂ indacene), 4.20 (d, *J* = 6.1 Hz, 2H, CH₂NH), 6.92 (s, 1H, CH_{arom.} indacene), 7.43 – 7.50 (m, 3H, CH_{arom.}, CONH), 7.87 – 7.91 (m, 2H, CH_{arom.}), 8.07 (s, 1H, NHCON), 10.55 (s, 1H, SO₂NH); ¹³C NMR (126 MHz, DMSO-*d*₆) δ 25.0 (CH₂ indacene), 28.2 (C(CH₃)₃), 30.0 (CH₂ indacene), 32.4 (CH₂ indacene), 43.0 (CH₂NH), 78.0 (C(CH₃)₃), 118.0, 127.2, 127.3, 128.5, 137.2, 138.3, 143.0, 145.9 (C_{arom.}), 148.9 (NHCONH), 155.7 (CONH); LC-MS (ESI) (90% H₂O to 100% MeOH in 10 min, then 100% MeOH to 20 min, DAD 220-400 nm), *t*_R = 11.20 min, 100% purity, *m/z* [M + H]⁺ calcd for C₂₅H₃₁N₃O₅S 486.21, found 486.4.

Thiophene-3-carboxylic acid (6)

Compound **6** was purchased from Sigma Aldrich/Merck (Darmstadt, Germany).

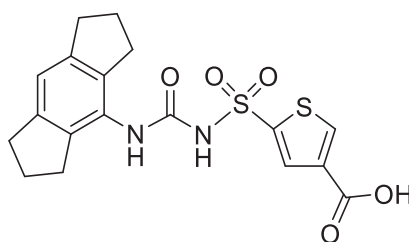
5-Sulfamoylthiophene-3-carboxylic acid (7)²³⁹

Compound **7** was prepared similar to a previously reported procedure.²³⁹ Chlorosulfonic acid (3.50 g, 30.0 mmol) was stirred at 0 °C under argon. Thiophene-3-carboxylic acid (**6**, 641 mg, 5.00 mmol) was added and stirred for 30 min at 0 °C under argon. The reaction mixture was heated to 60 °C for 3 h. The mixture was carefully poured onto crushed ice (100 g). The

resulting mixture was extracted with CH₂Cl₂ (3 × 50 mL). The combined organic layers were dried over Na₂SO₄, filtered and concentrated. The crude residue was dissolved in MeCN (10 mL) and concentrated aqueous ammonium hydroxide solution was added (25 mL). The reaction mixture was stirred for 1 h at 50 °C. After cooling to room temperature, the organic phase was evaporated *in vacuo* and the remaining aqueous solution was acidified with 2 N HCl to pH~1. The precipitate was collected, washed with 2 N HCl (5 mL) and hexane (5 mL) and dried under high vacuum to yield a white solid (131 mg).

Yield 13%; mp: 211-213 °C, lit.²³⁹ mp: 217-220 °C; ¹H NMR (600 MHz, DMSO-*d*₆) δ 7.78 (d, *J* = 1.5 Hz, 1H, CH_{arom.}), 7.79 (s, 2H, SO₂NH₂), 8.47 (d, *J* = 1.5 Hz, 1H, CH_{arom.}), 13.11 (s, 1H, COOH); ¹³C NMR (151 MHz, DMSO-*d*₆) δ 129.6, 133.7, 137.1, 146.6 (C_{arom.}), 162.7 (COOH); LC-MS (ESI) (90% H₂O to 100% MeCN in 10 min, then 100% MeCN to 15 min, DAD 220-800 nm), *t*_R = 0.34 min, 100% purity, *m/z* [M + Na]⁺ calcd for C₅H₅NO₄S₂ 229.96, found 229.9.

5-(*N*-((1,2,3,5,6,7-Hexahydro-*s*-indacen-4-yl)carbamoyl)sulfamoyl)thiophene-3-carboxylic acid (8)

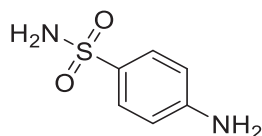


Triphosgene (237 mg, 800 μmol) was dissolved in dry THF (15 mL). The mixture was stirred for 30 min at 0 °C under nitrogen. TEA (162 mg, 1.60 mmol) was added. 1,2,3,5,6,7-Hexahydro-*s*-indacen-4-amine (**1**, 139 mg, 800 μmol) was dissolved in dry THF (15 mL) and dropwise added over 30 min. The mixture was stirred for additional 30 min at room temperature and then for 30 min at 75 °C. The solvent was evaporated *in vacuo* and the residue was taken up in dry THF (15 mL), filtered and concentrated to yield the intermediate 4-isocyanato-1,2,3,5,6,7-hexahydro-*s*-indacene (**2**). 5-Sulfamoylthiophene-3-carboxylic acid (**7**, 124 mg, 600 μmol) was dissolved in dry THF (8 mL). NaH (60% dispersion in mineral oil, 58 mg, 1.44 mmol) was added and the reaction mixture was stirred at 0 °C under nitrogen for 30 min. The crude intermediate **2** was dissolved in dry THF (5 mL) and added. The reaction mixture was stirred for 2 h at room temperature under nitrogen. The organic solvent was evaporated *in vacuo*. The residue was dispersed in H₂O (15 mL) and acidified with 2 N HCl. The precipitate was

collected, washed with EtOAc (2×5 mL) and dried under high vacuum to yield a beige solid (100 mg).

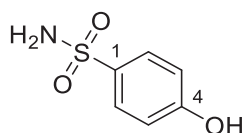
Yield 41%; mp: >230 °C; $^1\text{H NMR}$ (600 MHz, DMSO- d_6) δ 1.94 (quint, $J = 7.4$ Hz, 4H, CH_2 indacene), 2.58 (t, $J = 7.4$ Hz, 4H, CH_2 indacene), 2.79 (t, $J = 7.5$ Hz, 4H, CH_2 indacene), 6.95 (s, 1H, $\text{CH}_{\text{arom. indacene}}$), 7.95 (d, $J = 1.6$ Hz, 1H, $\text{CH}_{\text{arom. thiophene}}$), 8.32 (s, 1H, NHCON), 8.61 (d, $J = 1.6$ Hz, 1H, $\text{CH}_{\text{arom. thiophene}}$), 13.20 (s, 1H, COOH), one signal (1H, SO_2NH) is missing due to proton exchange; $^{13}\text{C NMR}$ (151 MHz, DMSO- d_6) δ 25.0, 30.1, 32.4 (CH_2 indacene), 118.1, 128.5, 132.8, 133.5, 137.3, 139.5, 141.5, 143.1 ($\text{C}_{\text{arom.}}$), 149.0 (NHCONH), 162.4 (COOH); LC-MS (ESI) (90% H_2O + 0.1% AcOH to 100% MeCN + 0.1% AcOH in 10 min, then 100% MeCN + 0.1% AcOH to 20 min, DAD 200-400 nm), $t_R = 8.49$ min, 83% purity, m/z $[\text{M} + \text{H}]^+$ calcd for $\text{C}_{18}\text{H}_{18}\text{N}_2\text{O}_5\text{S}_2$ 407.07, found 407.3.

4-Aminobenzenesulfonamide (9)



Compound **9** was purchased from TCI (Eschborn, Germany).

4-Hydroxybenzenesulfonamide (10)^{240,320}

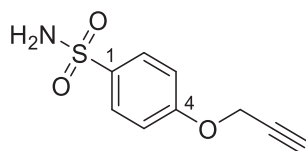


Compound **10** was prepared similar to a previously reported procedure.²⁴⁰ 4-Aminobenzenesulfonamide (**9**, 2.58 g, 15.0 mmol) was dispersed in a mixture of H_2O (20 mL) and concentrated H_2SO_4 (10 mL) and cooled to 0 °C. A solution of NaNO_2 (1.03 g, 15.0 mmol) in H_2O (10 mL) was added. The reaction mixture was stirred at 90 °C for 2 h. The reaction mixture was allowed to cool to room temperature and was placed in the freezer overnight. The precipitate was collected, washed with H_2O (10 mL) and dried under vacuum. The crude product was purified by silica gel column chromatography using $\text{CH}_2\text{Cl}_2/\text{MeOH}$ (19+1) as eluent to yield a beige solid (1.22 g).

Yield 47%; mp: 171-173 °C, lit.²⁴⁰ mp: 175 °C; $^1\text{H NMR}$ (500 MHz, DMSO- d_6) δ 6.85 – 6.89 (m, 2H, $\text{CH}_{\text{arom.}}$), 7.07 (s, 2H, SO_2NH_2), 7.63 – 7.66 (m, 2H, $\text{CH}_{\text{arom.}}$), 10.21 (s, 1H, OH); ^{13}C

NMR (126 MHz, DMSO-*d*₆) δ 115.2, 127.8 (C-2, C-3, C-5, C-6), 134.6 (C-1), 160.3 (C-4); **LC-MS** (ESI) (90% H₂O to 100% MeOH in 10 min, then 100% MeOH to 20 min, DAD 220-400 nm), t_R = 1.22 min, 98% purity, m/z [M + H]⁺ calcd for C₆H₇NO₃S 174.02, found 173.8.

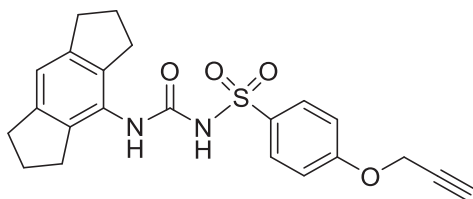
4-(Prop-2-yn-1-yloxy)benzenesulfonamide (11)^{241,321}



Compound **11** was prepared similar to a previously reported procedure.³²¹ 4-Hydroxybenzenesulfonamide (**10**, 693 mg, 4.00 mmol) was dissolved in dry DMF (5 mL) under nitrogen. K₂CO₃ (1.11 g, 8.00 mmol) was added. Propargyl bromide (80% solution in toluene, 595 mg, 4.00 mmol) was added and the reaction mixture was stirred at room temperature for 18 h under nitrogen. The reaction was quenched by the addition of H₂O (30 mL). The aqueous phase was extracted with EtOAc (3 × 50 mL). The combined organic layers were washed with brine (50 mL), dried over Na₂SO₄, filtered and concentrated. The residue was purified by silica gel column chromatography using petroleum ether/EtOAc (1+1) as eluent to yield a white solid (572 mg).

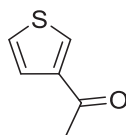
Yield 68%; mp: 144-146 °C, lit.³²¹ mp: 131-132 °C; **¹H NMR** (600 MHz, DMSO-*d*₆) δ 3.60 (t, J = 2.4 Hz, 1H, CCH), 4.90 (d, J = 2.4 Hz, 2H, CH₂), 7.12 – 7.15 (m, 2H, CH_{arom.}), 7.21 (s, 2H, SO₂NH₂), 7.75 – 7.78 (m, 2H, CH_{arom.}); **¹³C NMR** (151 MHz, DMSO-*d*₆) δ 55.7 (OCH₂), 78.6, 78.7 (CCH, CCH), 114.9, 127.6 (C-2, C-3, C-5, C-6), 136.9 (C-1), 159.4 (C-4); **LC-MS** (ESI) (90% H₂O to 100% MeOH in 10 min, then 100% MeOH to 20 min, DAD 220-400 nm), t_R = 7.05 min, 98% purity, m/z [M + H]⁺ calcd for C₉H₉NO₃S 212.04, found 212.0.

***N*-((1,2,3,5,6,7-Hexahydro-*s*-indacen-4-yl)carbamoyl)-4-(prop-2-yn-1-yloxy)benzenesulfonamide (**12**)**

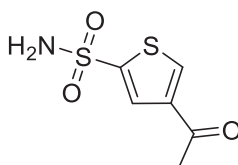


Triphosgene (237 mg, 800 μ mol) was dissolved in dry THF (15 mL). The mixture was stirred for 30 min at 0 °C under nitrogen. TEA (162 mg, 1.60 mmol) was added. 1,2,3,5,6,7-Hexahydro-*s*-indacen-4-amine (**1**, 139 mg, 800 μ mol) was dissolved in dry THF (30 mL) and dropwise added over 30 min. The mixture was stirred for additional 30 min at room temperature and then for 30 min at 75 °C. The solvent was evaporated *in vacuo* and the residue was taken up in dry THF (10 mL), filtered and concentrated *in vacuo* to yield the intermediate 4-isocyanato-1,2,3,5,6,7-hexahydro-*s*-indacene (**2**). 4-(Prop-2-yn-1-yloxy)benzenesulfonamide (**11**, 169 mg, 800 μ mol) was dissolved in dry THF (7 mL). NaH (60% dispersion in mineral oil, 58 mg, 1.44 mmol) was added and the reaction mixture was stirred at 0 °C under nitrogen for 30 min. The crude intermediate **2** was dissolved in dry THF (15 mL) and added. The reaction mixture was stirred for 2 h at room temperature under nitrogen. The organic solvent was evaporated *in vacuo*. The residue was dispersed in H₂O (30 mL) and acidified with 2 N HCl. The precipitate was collected and dried under high vacuum to yield a beige solid (276 mg).

Yield 84%; mp: decomposition >186 °C; ¹H NMR (600 MHz, DMSO-*d*₆) δ 1.92 (quint, *J* = 7.4 Hz, 4H, CH₂ indacene), 2.53 (t, *J* = 7.4 Hz, 4H, CH₂ indacene), 2.77 (t, *J* = 7.4 Hz, 4H, CH₂ indacene), 3.61 – 3.63 (m, 1H, CCH), 4.92 (d, *J* = 2.0 Hz, 2H, OCH₂), 6.92 (s, 1H, CH_{arom.} indacene), 7.18 (d, *J* = 8.5 Hz, 2H), 7.89 (d, *J* = 8.7 Hz, 2H, CH_{arom.}), 8.06 (s, 1H, NHCON), 10.62 (s, 1H, SO₂NH); ¹³C NMR (151 MHz, DMSO-*d*₆) δ 25.0, 30.0, 32.4 (CH₂ indacene), 55.9 (OCH₂), 78.4, 78.8 (CCH, CCH), 114.9, 117.9, 128.6, 129.5, 132.3, 137.1, 143.0 (C_{arom.}), 149.0 (NHCONH), 160.6 (C_{arom.}); LC-MS (ESI) (90% H₂O to 100% MeCN in 10 min, then 100% MeCN to 15 min, DAD 220-600 nm), *t*_R = 5.93 min, 98% purity, *m/z* [M + H]⁺ calcd for C₂₂H₂₂N₂O₄S 411.14, found 411.2.

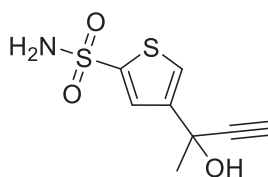
1-(Thiophen-3-yl)ethan-1-one (13)

Compound **13** was purchased from abcr (Karlsruhe, Germany).

4-Acetylthiophene-2-sulfonamide (14)³²²

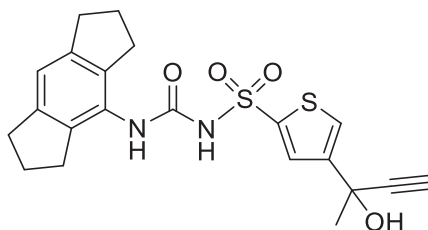
Chlorosulfonic acid (10.5 g, 90.0 mmol) was stirred at 0 °C under argon. 1-(Thiophen-3-yl)ethan-1-one (**13**, 1.89 g, 15.0 mmol) was added carefully. The mixture was stirred for 30 min at 0 °C under argon. The reaction mixture was heated to 60 °C for 3 h. The mixture was allowed to cool to room temperature and was carefully poured onto crushed ice. The resulting mixture was extracted with CH₂Cl₂ (3 × 50 mL). The combined organic layers were dried over Na₂SO₄, filtered and concentrated *in vacuo*. The crude intermediate was dissolved in MeCN (30 mL) and ammonia gas was added (1 atm, balloon). The reaction mixture was stirred for 1 h at room temperature. The solvent was evaporated *in vacuo*. The precipitate was collected, washed with water (3 × 20 mL) and hexane (3 × 20 mL). The residue was purified by silica gel column chromatography using petroleum ether/EtOAc (1+1) as eluent to yield a white solid (386 mg).

Yield 13%; mp: 124-126 °C, lit.³²² mp: 132-134 °C; ¹H NMR (500 MHz, DMSO-*d*₆) δ 2.51 (s, 3H, CH₃), 7.79 (s, 2H, SO₂NH₂), 7.83 (d, *J* = 1.6 Hz, 1H, CH_{arom.}), 8.66 (d, *J* = 1.6 Hz, 1H, CH_{arom.}); ¹³C NMR (126 MHz, DMSO-*d*₆) δ 27.3 (CH₃), 128.3, 137.4, 141.2, 146.8 (C_{arom.}), 191.9 (CO); LC-MS (ESI) (90% H₂O to 100% MeOH in 10 min, then 100% MeOH to 20 min, DAD 220-400 nm), *t*_R = 4.18 min, 96% purity, *m/z* [M + H]⁺ calcd for C₆H₇NO₃S₂ 205.99, found 205.9.

4-(2-Hydroxybut-3-yn-2-yl)thiophene-2-sulfonamide (15)

Ethynylmagnesium bromide (0.5 M in THF, 45 mL, 22.5 mmol) was stirred at 0 °C under nitrogen. 4-Acetylthiophene-2-sulfonamide (**14**, 924 mg, 4.50 mmol) was dissolved in dry THF (10 mL) and dropwise added at 0 °C. The reaction mixture was stirred for 2 h at room temperature. The reaction was quenched by the addition of saturated NH₄Cl solution (30 mL). The mixture was extracted with CH₂Cl₂ (3 × 50 mL). The combined organic layers were washed with brine (50 mL), dried over NaSO₄, filtered and concentrated *in vacuo*. The residue was purified by silica gel flash column chromatography using a CH₂Cl₂/MeOH gradient (0% to 6.5% MeOH) to yield a colorless resin (461 mg).

Yield 44%; ¹H NMR (500 MHz, DMSO-*d*₆) δ 1.65 (s, 3H, CH₃), 3.53 (s, 1H, CCH), 6.22 (s, 1H, OH), 7.58 (d, *J* = 1.6 Hz, 1H, CH_{arom.}), 7.64 (s, 2H, SO₂NH₂), 7.67 (d, *J* = 1.7 Hz, 1H, CH_{arom.}); ¹³C NMR (126 MHz, DMSO-*d*₆) δ 32.2 (CH₃), 65.2, 74.0, 87.8 (COH, CCH, CCH), 125.1, 128.4, 145.7, 147.8 (C_{arom.}); LC-MS (ESI) (90% H₂O to 100% MeCN in 10 min, then 100% MeCN to 15 min, DAD 200-600 nm), *t*_R = 1.58 min, 99% purity, *m/z* [M - H]⁻ calcd for C₈H₉NO₃S₂ 229.99, found 230.0.

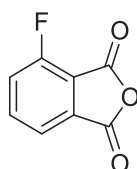
***N*-((1,2,3,5,6,7-Hexahydro-*s*-indacen-4-yl)carbamoyl)-4-(2-hydroxybut-3-yn-2-yl)thiophene-2-sulfonamide (16)**

Triphosgene (712 mg, 2.4 mmol) was dissolved in dry THF (15 mL). The mixture was stirred for 30 min at 0 °C under nitrogen. TEA (405 mg, 4.00 mmol) was added. 1,2,3,5,6,7-Hexahydro-*s*-indacen-4-amine (**1**, 416 mg, 2.40 mmol) was dissolved in dry THF (30 mL) and dropwise added over 30 min. The mixture was stirred for additional 30 min at room temperature and then for 30 min at 75 °C. The solvent was evaporated *in vacuo* and the residue was taken up in dry THF (10 mL), filtered and concentrated *in vacuo* to yield the intermediate 4-

isocyanato-1,2,3,5,6,7-hexahydro-*s*-indacene (**2**). 4-(2-Hydroxybut-3-yn-2-yl)thiophene-2-sulfonamide (**15**, 463 mg, 2.00 mmol) was dissolved in dry THF (7 mL). NaH (60% dispersion in mineral oil, 144 mg, 3.60 mmol) was added and the reaction mixture was stirred at 0 °C under nitrogen for 30 min. The crude intermediate 4-isocyanato-1,2,3,5,6,7-hexahydro-*s*-indacene was dissolved in dry THF (15 mL) and added. The reaction mixture was stirred for 2 h at room temperature under nitrogen. The organic solvent was evaporated *in vacuo*. The residue was dispersed in H₂O (30 mL) and acidified with 2 N HCl. The precipitate was collected and dried under high vacuum. The crude product was purified by silica gel column chromatography using CH₂Cl₂/MeOH (19+1) as eluent to yield a beige solid (266 mg).

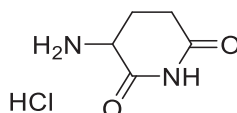
Yield 31%; mp: decomposition >133 °C; ¹H NMR (600 MHz, DMSO-*d*₆) δ 1.64 (s, 3H, CH₃), 1.94 (quint, *J* = 7.4 Hz, 4H), 2.60 (t, *J* = 7.4 Hz, 4H), 2.79 (t, *J* = 7.4 Hz, 4H, CH₂ indacene), 3.55 (s, 1H, CCH), 6.28 (s, 1H, OH), 6.94 (s, 1H, CH_{arom.} indacene), 7.77 (d, *J* = 1.7 Hz, 1H), 7.84 (d, *J* = 1.7 Hz, 1H, CH_{arom.} thiophene), 8.15 (s, 1H, CONH), 10.64 (s, 1H, SO₂NH); ¹³C NMR (151 MHz, DMSO-*d*₆) δ 25.0, 30.1, 32.2, 32.4 (CH₂ indacene, CH₃), 65.2, 74.1, 87.7 (COH, CCH, CCH), 118.0, 127.9, 128.6, 131.3, 137.3, 140.7, 143.1, 147.9 (C_{arom.}), 149.0 (CO); LC-MS (ESI) (90% H₂O to 100% MeCN in 10 min, then 100% MeCN to 15 min, DAD 200-600 nm), *t*_R = 4.11 min, 96% purity, *m/z* [M + H]⁺ calcd for C₂₁H₂₂N₂O₄S₂ 431.11, found 431.1.

4-Fluoroisobenzofuran-1,3-dione (**17**)

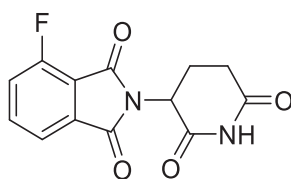


Compound **17** was purchased from BLDpharm (Kaiserslautern, Germany).

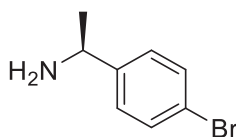
3-Aminopiperidine-2,6-dione hydrochloride (**18**)



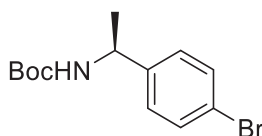
Compound **18** was purchased from BLDpharm (Kaiserslautern, Germany).

2-(2,6-Dioxopiperidin-3-yl)-4-fluoroisoindoline-1,3-dione (19)^{208,247,248}

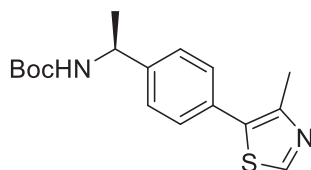
Compound **19** was prepared as described before.³²³

(S)-1-(4-Bromophenyl)ethan-1-amine (20)

Compound **20** was purchased from abcr (Karlsruhe, Germany).

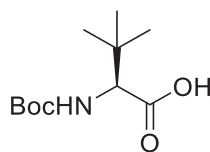
***tert*-Butyl (S)-(1-(4-bromophenyl)ethyl)carbamate (21)**²¹⁰

Compound **21** was prepared as described before.³²³

***tert*-Butyl (S)-(1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)carbamate (22)**²¹⁰

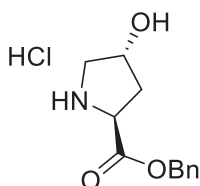
Compound **22** was prepared as described before.³²³

(S)-2-((tert-Butoxycarbonyl)amino)-3,3-dimethylbutanoic acid (23)



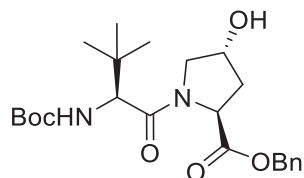
Compound **23** was purchased from abcr (Karlsruhe, Germany).

Benzyl (2S,4R)-4-hydroxypyrrolidine-2-carboxylate hydrochloride (24)



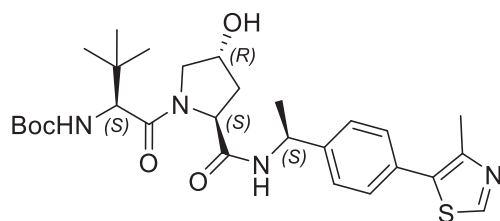
Compound **24** was purchased from abcr (Karlsruhe, Germany).

Benzyl (2S,4R)-1-((S)-2-((tert-butoxycarbonyl)amino)-3,3-dimethylbutanoyl)-4-hydroxypyrrolidine-2-carboxylate (25)³²⁴

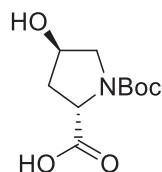


Compound **25** was prepared as described before.³²³

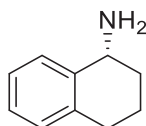
tert-Butyl ((S)-1-((2S,4R)-4-hydroxy-2-(((S)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)carbamoyl)pyrrolidin-1-yl)-3,3-dimethyl-1-oxobutan-2-yl)carbamate (26)^{209,210,255,256,325}



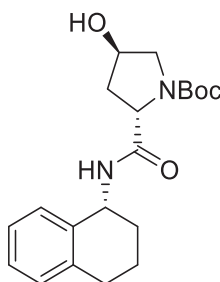
Compound **26** was prepared as described before.³²³

(2*S*,4*R*)-1-(*tert*-Butoxycarbonyl)-4-hydroxypyrrolidine-2-carboxylic acid (27)

Compound **27** was purchased from BLDpharm (Kaiserslautern, Germany).

(*R*)-1,2,3,4-Tetrahydronaphthalen-1-amine (28)

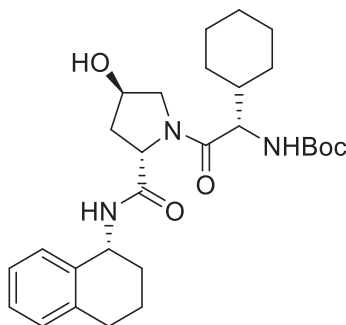
Compound **28** was purchased from Sigma Aldrich/Merck (Darmstadt, Germany).

***tert*-Butyl (2*S*,4*R*)-4-hydroxy-2-(((*R*)-1,2,3,4-tetrahydronaphthalen-1-yl)carbamoyl)pyrrolidine-1-carboxylate (29)²⁷⁴**

Compound **29** was prepared similar to a previously reported procedure.²⁷⁴ Boc-Hyp-OH (**27**, 11.6 g, 50.0 mmol) was dissolved in dry CH₂Cl₂ (150 mL) at 0 °C under argon. HOBT hydrate (8.42 g, 55.0 mmol), TEA (5.57 g, 55 mmol) and EDC × HCl (10.5 g, 55.0 mmol) were added. The mixture was stirred at 0 °C under argon for 15 min. (*R*)-1,2,3,4-Tetrahydronaphthalen-1-amine (**28**, 7.36 g, 50.0 mmol) was added. The reaction mixture was stirred for 18 h at room temperature under argon. The reaction was quenched by the addition of 2 N HCl (100 mL). The organic layer was separated and the aqueous phase was extracted with EtOAc (2 × 200 mL). The combined organic layers were washed with water (100 mL) and brine (100 mL). The organic layer was dried over Na₂SO₄, filtered and concentrated *in vacuo*. The crude product was purified by silica gel column chromatography using a petroleum ether/EtOAc gradient (50% to 100% EtOAc) as eluent to yield a white solid (15.3 g).

Yield 85%; mp: 48-54 °C; $^1\text{H NMR}$ (500 MHz, $\text{DMSO-}d_6$) δ 1.33 – 1.43 (m, 9H), 1.60 – 1.77 (m, 2H), 1.78 – 1.94 (m, 3H), 1.96 – 2.13 (m, 1H), 2.65 – 2.80 (m, 2H), 3.37 – 3.48 (m, 1H), 4.17 – 4.30 (m, 2H), 4.90 – 4.97 (m, 2H), 7.03 – 7.30 (m, 4H), 8.15 – 8.21 (m, 1H), one signal (1H) is obscured by solvent signal; $^{13}\text{C NMR}$ (126 MHz, $\text{DMSO-}d_6$) δ 19.9, 28.0, 28.7, 29.6, 38.5, 46.4, 54.7, 58.6, 67.7, 78.4, 125.5, 126.6, 128.2, 128.6, 137.0, 137.4, 153.6, 171.9; **LC-MS** (ESI) (90% H_2O to 100% MeCN in 10 min, then 100% MeCN to 15 min, DAD 220-600 nm), t_R = 5.64 min, 71% purity, m/z $[\text{M} + \text{H}]^+$ calcd for $\text{C}_{20}\text{H}_{28}\text{N}_2\text{O}_4$ 361.21, found 361.2.

***tert*-Butyl ((*S*)-1-cyclohexyl-2-((2*S*,4*R*)-4-hydroxy-2-(((*R*)-1,2,3,4-tetrahydronaphthalen-1-yl)carbamoyl)pyrrolidin-1-yl)-2-oxoethyl)carbamate (30)**²⁷⁴



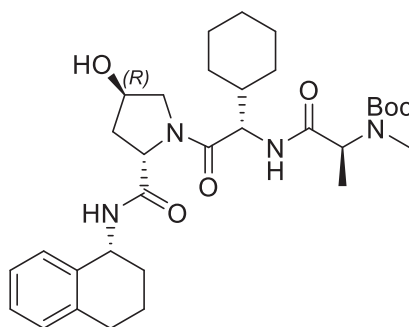
Compound **30** was prepared similar to a previously reported procedure.²⁷⁴ *tert*-Butyl (2*S*,4*R*)-4-hydroxy-2-(((*R*)-1,2,3,4-tetrahydronaphthalen-1-yl)carbamoyl)pyrrolidine-1-carboxylate (**29**, 14.4 g, 40.0 mmol) was stirred in 1 N HCl in EtOAc (100 mL) at room temperature for 18 h. The precipitate was filtered off, washed with dry EtOAc (2 × 15 mL) and dried under high vacuum to yield the intermediate (2*S*,4*R*)-4-hydroxy-2-(((*R*)-1,2,3,4-tetrahydronaphthalen-1-yl)carbamoyl)pyrrolidin-1-ium chloride as a white solid (10.6 g, 35.7 mmol). Yield 89%; $^1\text{H NMR}$ (500 MHz, $\text{DMSO-}d_6$) δ 1.66 – 1.79 (m, 2H), 1.80 – 1.93 (m, 3H), 2.25 – 2.33 (m, 1H), 2.65 – 2.82 (m, 2H), 3.07 – 3.15 (m, 1H), 4.29 – 4.37 (m, 1H), 4.40 – 4.46 (m, 1H), 4.92 – 5.03 (m, 1H), 5.55 (s, 1H), 7.07 – 7.25 (m, 4H), 8.68 (s, 1H), 8.98 (d, J = 8.4 Hz, 1H), 10.21 (s, 1H), one signal (1H) is obscured by solvent signal; $^{13}\text{C NMR}$ (126 MHz, $\text{DMSO-}d_6$) δ 19.5, 28.6, 29.4, 38.8, 47.0, 53.3, 57.9, 69.0, 125.8, 127.0, 128.4, 128.8, 136.3, 137.1, 167.2; **LC-MS** (ESI) (90% H_2O to 100% MeCN in 10 min, then 100% MeCN to 15 min, DAD 220-600 nm), t_R = 4.04 min, 88% purity, m/z $[\text{M} + \text{H}]^+$ calcd for $\text{C}_{15}\text{H}_{20}\text{N}_2\text{O}_2$ 261.16, found 261.2.

Boc-Chg-OH (10.8 g, 42.0 mmol) was dissolved in dry DMF (150 mL) at 0° C under argon. HOBt hydrate (6.43 g, 42.0 mmol), DIPEA (5.43 g, 42.0 mmol) and EDC × HCl (8.05 g, 42.0 mmol) were added. The mixture was stirred at 0 °C under argon for 15 min. The deprotected intermediate (10.4 g, 35.0 mmol) was added. The reaction mixture was stirred for

18 h at room temperature under argon. The mixture was concentrated *in vacuo*. The residue was diluted with H₂O (150 mL) and was extracted with EtOAc (3 × 100 mL). The combined organic layers were washed with 1 N HCl (100 mL), saturated NaHCO₃ solution (100 mL) and brine (100 mL). The organic layer was dried over Na₂SO₄, filtered and concentrated *in vacuo*. The crude product was purified by silica gel column chromatography using a petroleum ether/EtOAc gradient (50% to 66% EtOAc) as eluent to yield a white solid (14.2 g).

Yield 81%; mp: 80-86 °C; ¹H NMR (500 MHz, DMSO-*d*₆) δ 0.89 – 1.16 (m, 5H), 1.36 (s, 9H), 1.56 – 1.73 (m, 7H), 1.75 – 1.93 (m, 4H), 1.97 – 2.03 (m, 1H), 2.65 – 2.79 (m, 2H), 3.48 – 3.73 (m, 2H), 4.04 – 4.10 (m, 1H), 4.31 – 4.44 (m, 2H), 4.89 – 4.94 (m, 1H), 5.06 (d, *J* = 3.7 Hz, 1H), 6.63 (d, *J* = 8.7 Hz, 1H), 7.04 – 7.16 (m, 3H), 7.25 – 7.32 (m, 1H), 8.17 (d, *J* = 8.8 Hz, 1H); ¹³C NMR (126 MHz, DMSO-*d*₆) δ 20.3, 25.6, 25.7, 25.9, 27.8, 28.1, 28.7, 28.8, 29.9, 37.8, 46.4, 55.4, 56.6, 58.8, 68.8, 77.9, 125.6, 126.5, 128.2, 128.4, 136.8, 137.6, 155.4, 170.2, 170.9; LC-MS (ESI) (90% H₂O to 100% MeCN in 10 min, then 100% MeCN to 15 min, DAD 220-600 nm), *t*_R = 7.34 min, 100% purity, *m/z* [M + H]⁺ calcd for C₂₈H₄₁N₃O₅ 500.31, found 500.5.

***tert*-Butyl ((*S*)-1-(((*S*)-1-cyclohexyl-2-((2*S*,4*R*)-4-hydroxy-2-(((*R*)-1,2,3,4-tetrahydronaphthalen-1-yl)carbamoyl)pyrrolidin-1-yl)-2-oxoethyl)amino)-1-oxopropan-2-yl)(methyl)carbamate (31)**²⁷⁴



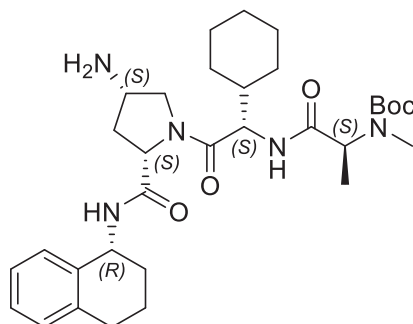
Compound **31** was prepared similar to a previously reported procedure.²⁷⁴ *tert*-Butyl ((*S*)-1-cyclohexyl-2-((2*S*,4*R*)-4-hydroxy-2-(((*R*)-1,2,3,4-tetrahydronaphthalen-1-yl)carbamoyl)pyrrolidin-1-yl)-2-oxoethyl)carbamate (**30**, 14.0 g, 28.0 mmol) was stirred in 1 N HCl in EtOAc (70 mL) at room temperature for 18 h. The precipitate was filtered off, washed with dry EtOAc (2 × 15 mL) and dried under high vacuum to yield the intermediate (*S*)-1-cyclohexyl-2-((2*S*,4*R*)-4-hydroxy-2-(((*R*)-1,2,3,4-tetrahydronaphthalen-1-yl)carbamoyl)pyrrolidin-1-yl)-2-oxoethan-1-aminium chloride as a white solid (10.6 g, 24.3 mmol). Yield 87%; ¹H NMR (500 MHz, DMSO-*d*₆) δ 1.12 – 1.24 (m, 5H), 1.57 – 1.65

(m, 2H), 1.67 – 1.76 (m, 5H), 1.79 – 1.90 (m, 5H), 2.05 – 2.12 (m, 1H), 2.66 – 2.79 (m, 2H), 3.57 – 3.63 (m, 1H), 3.66 – 3.71 (m, 1H), 3.93 – 3.99 (m, 1H), 4.35 – 4.40 (m, 1H), 4.47 (t, J = 8.2 Hz, 1H), 4.90 – 4.96 (m, 1H), 7.06 – 7.16 (m, 3H), 7.29 (d, J = 7.4 Hz, 1H), 8.14 – 8.22 (m, 3H), 8.37 (d, J = 8.7 Hz, 1H); ^{13}C NMR (126 MHz, DMSO- d_6) δ 20.3, 25.4, 25.5, 25.7, 26.9, 27.7, 28.7, 29.9, 38.0, 46.5, 55.2, 55.7, 59.0, 68.9, 125.6, 126.5, 128.1, 128.5, 136.9, 137.6, 166.9, 170.5; LC-MS (ESI) (90% H₂O to 100% MeCN in 10 min, then 100% MeCN to 15 min, DAD 220-600 nm), t_R = 5.38 min, 97% purity, m/z [M + H]⁺ calcd for C₂₃H₃₃N₃O₃ 400.26, found 400.3.

Boc-*N*-Me-L-Ala-OH (5.85 g, 28.8 mmol) was dissolved in dry DMF (100 mL) at 0° C under argon. HOBT hydrate (4.41 g, 28.8 mmol), DIPEA (3.72 g, 28.8 mmol) and EDC × HCl (5.52 g, 28.8 mmol) were added. The mixture was stirred at 0 °C under argon for 15 min. The deprotected intermediate (10.5 g, 24.0 mmol) was added. The reaction mixture was stirred for 18 h at room temperature under argon. The mixture was concentrated *in vacuo*. The residue was diluted with H₂O (100 mL) and was extracted with EtOAc (3 × 100 mL). The combined organic layers were washed with 1 N HCl (70 mL), saturated NaHCO₃ solution (70 mL) and brine (70 mL). The organic layer was dried over Na₂SO₄, filtered and concentrated *in vacuo*. The crude product was purified by silica gel column chromatography using EtOAc as eluent to yield **9** as a white solid (9.91 g).

Yield 71%; mp: 74-80 °C; ^1H NMR (500 MHz, DMSO- d_6) δ 0.90 – 1.23 (m, 8H), 1.42 (s, 9H), 1.56 – 1.75 (m, 7H), 1.77 – 1.94 (m, 4H), 1.99 – 2.05 (m, 1H), 2.66 – 2.79 (m, 5H), 3.52 – 3.60 (m, 1H), 3.64 – 3.72 (m, 1H), 4.33 – 4.44 (m, 3H), 4.50 – 4.61 (m, 1H), 4.87 – 4.97 (m, 1H), 5.00 – 5.11 (m, 1H), 7.03 – 7.17 (m, 3H), 7.30 (d, J = 7.5 Hz, 1H), 7.43 – 7.78 (m, 1H), 8.21 (d, J = 8.8 Hz, 1H); ^{13}C NMR (126 MHz, DMSO- d_6) δ 15.5, 20.4, 25.5, 25.7, 25.8, 27.6, 28.0, 28.7, 28.9, 29.9, 37.8, 46.5, 53.5, 54.5, 55.5, 58.7, 68.8, 79.0, 125.6, 126.5, 128.2, 128.4, 136.8, 137.7, 154.9, 169.6, 170.9, one signal (CO) is missing; LC-MS (ESI) (90% H₂O to 100% MeCN in 10 min, then 100% MeCN to 15 min, DAD 220-600 nm), t_R = 7.51 min, 99% purity, m/z [M + H]⁺ calcd for C₃₂H₄₈N₄O₆ 585.36, found 585.5.

tert-Butyl ((S)-1-(((S)-2-((2S,4S)-4-amino-2-(((R)-1,2,3,4-tetrahydronaphthalen-1-yl)carbamoyl)pyrrolidin-1-yl)-1-cyclohexyl-2-oxoethyl)amino)-1-oxopropan-2-yl)(methyl)carbamate (32)³²⁶

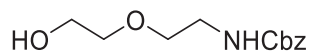


tert-Butyl ((S)-1-(((S)-1-cyclohexyl-2-((2S,4R)-4-hydroxy-2-(((R)-1,2,3,4-tetrahydronaphthalen-1-yl)carbamoyl)pyrrolidin-1-yl)-2-oxoethyl)amino)-1-oxopropan-2-yl)(methyl)carbamate (31, 9.36 g, 16.0 mmol) was dissolved in dry CH₂Cl₂ (40 mL) and cooled to 0 °C. TEA (2.43 g, 24.0 mmol) and methanesulfonyl chloride (2.75 g, 24.0 mmol) were added. The reaction mixture was allowed to warm to room temperature and was stirred for 24 h. The reaction mixture was concentrated *in vacuo*. The residue was taken up in dry DMF (50 mL) under argon. NaN₃ (3.12 g, 48.0 mmol) was added. The reaction mixture was stirred for 72 h at 70 °C. The mixture was carefully concentrated. The residue was taken up in dry THF (70 mL) under argon. Triphenylphosphine (8.39 g, 32.0 mmol) was added and the reaction mixture was stirred for 1 h. Concentrated aqueous ammonium hydroxide (70 mL) was added and the reaction mixture was stirred for 18 h. The reaction mixture was concentrated *in vacuo* and the aqueous residue was diluted with brine (40 mL). The aqueous phase was extracted with EtOAc (3 × 100 mL). The combined organic layers were washed with water (100 mL), brine (100 mL), dried over Na₂SO₄, filtered and concentrated *in vacuo*. The crude product was purified by silica gel column chromatography using CH₂Cl₂/MeOH (9+1) as eluent to yield a white solid (7.11 g).

Yield 76%; mp: 67-73 °C; ¹H NMR (500 MHz, DMSO-*d*₆) δ 0.89 – 1.05 (m, 2H), 1.05 – 1.24 (m, 6H), 1.41 (s, 9H), 1.54 – 1.77 (m, 8H), 1.79 – 1.89 (m, 3H), 2.24 – 2.30 (m, 1H), 2.67 – 2.77 (m, 5H), 3.23 – 3.28 (m, 1H), 3.32 – 3.39 (m, 1H), 3.89 (dd, *J* = 9.7, 6.2 Hz, 1H), 4.20 – 4.27 (m, 1H), 4.35 (t, *J* = 8.0 Hz, 1H), 4.41 – 4.62 (m, 1H), 4.88 – 4.96 (m, 1H), 7.00 – 7.20 (m, 3H), 7.24 – 7.35 (m, 1H), 7.48 – 7.81 (m, 1H), 8.28 (d, *J* = 8.6 Hz, 1H), one signal (2H) is missing due to proton exchange; ¹³C NMR (126 MHz, DMSO-*d*₆) δ 15.4, 20.2, 25.5, 25.7, 25.8, 28.0, 28.7, 28.8, 29.8, 30.0, 37.8, 46.5, 51.0, 54.7, 55.4, 59.2, 79.0, 125.6, 126.5, 128.3, 128.5, 136.9, 137.4, 154.8, 169.6, 171.0, two signals are missing; LC-MS (ESI) (90% H₂O to 100%

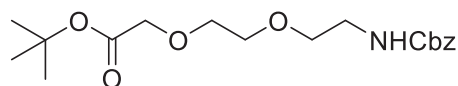
MeCN in 10 min, then 100% MeCN to 15 min, DAD 220-600 nm), $t_R = 7.96$ min, 100% purity, m/z $[M + H]^+$ calcd for $C_{32}H_{49}N_5O_5$ 584.38, found 584.6.

Benzyl (2-(2-hydroxyethoxy)ethyl)carbamate (33)^{229,249,279}



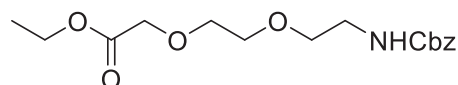
Compound **33** was prepared as described before.³⁰⁰

***tert*-Butyl 3-oxo-1-phenyl-2,7,10-trioxa-4-azadodecan-12-oate (34)**^{229,249,279}



Compound **34** was prepared as described before.³⁰⁰

Ethyl 3-oxo-1-phenyl-2,7,10-trioxa-4-azadodecan-12-oate (35)²⁴⁹

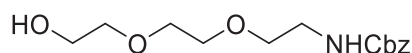


Compound **35** was prepared similar to a previously reported procedure.²⁴⁹ Benzyl (2-(2-hydroxyethoxy)ethyl)carbamate (**33**, 14.8 g, 62.0 mmol) was dissolved in dry toluene (80 mL) and it was cooled to 0 °C. A mixture of potassium *tert*-butoxide (10.4 g, 93.0 mmol) in *tert*-butyl alcohol (100 mL) was added and the reaction mixture was stirred for 30 min at 0 °C. Ethyl bromoacetate (20.7 g, 124 mmol) was added. The reaction mixture was stirred for 3 h at 0 °C and for 18 h at room temperature. The reaction was quenched by the addition of 1 N HCl (30 mL). The organic layer was separated, washed with brine (150 mL), dried over Na_2SO_4 , filtered and concentrated *in vacuo*. The crude product was purified via silica gel column chromatography using a gradient of petroleum ether/EtOAc (2+1) to petroleum ether/EtOAc (1+1) to yield a colorless oil (7.49 g).

Yield 37%; ¹H NMR (600 MHz, DMSO- d_6) δ 1.19 (t, $J = 7.1$ Hz, 3H, CH₃), 3.15 (q, $J = 5.9$ Hz, 2H), 3.41 (t, $J = 6.0$ Hz, 2H), 3.50 – 3.53 (m, 2H), 3.56 – 3.60 (m, 2H), 4.09 – 4.13 (m, 4H), 5.01 (s, 2H, CH₂), 7.20 – 7.27 (m, 1H), 7.29 – 7.38 (m, 5H, NH, CH_{arom.}); ¹³C NMR (151 MHz, DMSO- d_6) δ 14.0 (CH₃), 60.1, 65.2, 67.7, 69.1, 69.5, 69.9 (OCH₂), 127.7, 127.7, 128.3, 137.2 (C_{arom.}), 156.1 (NHCO), 170.1 (COO), one signal (NHCH₂) is obscured by solvent signal;

LC-MS (ESI) (90% H₂O to 100% MeOH in 10 min, then 100% MeOH to 20 min, DAD 205-400 nm), t_R = 10.02 min, 95% purity, m/z [M + H]⁺ calcd for C₁₆H₂₃NO₆ 326.16, found 326.2.

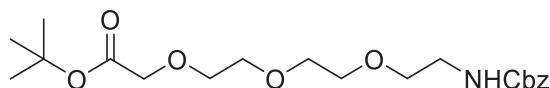
Benzyl (2-(2-(2-hydroxyethoxy)ethoxy)ethyl)carbamate (36)^{229,249}



Compound **36** was prepared similar to a previously reported procedure.²⁴⁹ Ethyl 3-oxo-1-phenyl-2,7,10-trioxa-4-azadodecan-12-oate (**35**, 6.51 g, 20.0 mmol) was dissolved and stirred in dry THF (100 mL). NaBH₄ (4.92 g, 130 mmol) was added in portions to the stirring solution over 15 min at reflux. The reaction mixture was allowed to cool to room temperature. MeOH (100 mL) was carefully added in portions over 30 min. The reaction mixture was stirred at reflux for 60 min. The solution was allowed to cool to room temperature. The reaction was quenched by the addition of saturated NH₄Cl solution (100 mL). The organic layer was separated and the aqueous layer was extracted with EtOAc (3 × 100 mL). The combined organic layers were washed with brine (50 mL), dried over Na₂SO₄, filtered and concentrated *in vacuo*. The crude product was purified by silica gel column chromatography using petroleum ether/EtOAc (1+2) as eluent to yield a colorless oil (3.21 g).

Yield 57%; ¹H NMR (600 MHz, DMSO-*d*₆) δ 3.15 (q, J = 5.9 Hz, 2H), 3.39 – 3.43 (m, 4H), 3.46 – 3.51 (m, 6H, CH₂), 4.55 (t, J = 5.5 Hz, 1H, OH), 5.01 (s, 2H, OCH₂), 7.19 – 7.28 (m, 1H), 7.29 – 7.43 (m, 5H, NH, CH_{arom.}); ¹³C NMR (151 MHz, DMSO-*d*₆) δ 40.2 (CH₂NH), 60.2 (CH₂OH), 65.2, 69.1, 69.6, 69.7, 72.3 (OCH₂), 127.7, 127.7, 128.3, 137.2 (C_{arom.}), 156.1 (CO); **LC-MS** (ESI) (90% H₂O to 100% MeOH in 10 min, then 100% MeOH to 20 min, DAD 200-400 nm), t_R = 8.68 min, 100% purity, m/z [M + H]⁺ calcd for C₁₄H₂₁NO₅ 284.15, found 283.9.

***tert*-Butyl 3-oxo-1-phenyl-2,7,10,13-tetraoxa-4-azapentadecan-15-oate (37)**^{229,249}

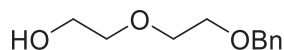


Compound **37** was prepared similar to a previously reported procedure.²⁴⁹ Benzyl (2-(2-(2-hydroxyethoxy)ethoxy)ethyl)carbamate (**36**, 6.23 g, 22.0 mmol) was dissolved in dry THF (50 mL) at 0 °C. A mixture of potassium *tert*-butoxide (3.29 g, 29.3 mmol) in dry THF (40 mL) was added and the reaction mixture was stirred for 30 min at 0 °C. *tert*-Butyl bromoacetate

(8.58 g, 44.0 mmol) was added. The reaction mixture was stirred for 3 h at 0 °C and for 18 h at room temperature. The reaction was quenched by the addition of H₂O (20 mL) and the mixture was concentrated *in vacuo*. The residue was partitioned between EtOAc (100 mL) and H₂O (100 mL). The aqueous layer was extracted with EtOAc (2 × 50 mL). The combined organic layers were washed with brine (50 mL), dried over Na₂SO₄, filtered and concentrated *in vacuo*. The residue was purified by silica gel column chromatography using petroleum ether/EtOAc (1+1) as eluent to yield a colorless oil (2.52 g).

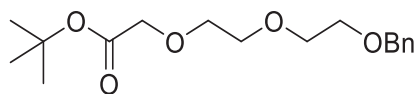
Yield 29%; ¹H NMR (600 MHz, DMSO-*d*₆) δ 1.41 (s, 9H, C(CH₃)₃), 3.12 – 3.18 (m, 2H), 3.41 (t, *J* = 6.0 Hz, 2H), 3.48 – 3.54 (m, 6H), 3.54 – 3.57 (m, 2H), 3.97 (s, 2H), 5.01 (s, 2H, CH₂), 7.19 – 7.27 (m, 1H), 7.28 – 7.39 (m, 5H, NH, CH_{arom.}); ¹³C NMR (151 MHz, DMSO-*d*₆) δ 27.7 (C(CH₃)₃), 40.2 (CH₂NH), 65.2, 68.1, 69.1, 69.5, 69.7, 69.7, 69.8 (OCH₂), 80.6 (C(CH₃)₃), 127.7, 127.7, 128.3, 137.2 (C_{arom.}), 156.1 (CONH), 169.3 (COO); LC-MS (ESI) (90% H₂O to 100% MeOH in 10 min, then 100% MeOH to 20 min, DAD 200-400 nm), *t*_R = 10.72 min, 93% purity, *m/z* [M + H]⁺ calcd for C₂₀H₃₁NO₇ 398.22, found 398.2.

2-(2-(Benzyloxy)ethoxy)ethan-1-ol (**38**)



Compound **38** was purchased from TCI (Eschborn, Germany).

tert-Butyl 2-(2-(2-(benzyloxy)ethoxy)ethoxy)acetate (**39**)²⁸⁰

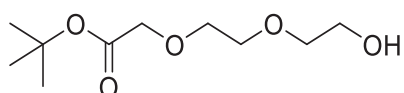


Compound **39** was prepared similar to a previously reported procedure.²⁸⁰ 2-(2-(Benzyloxy)ethoxy)ethan-1-ol (**38**, 3.02 g, 15.4 mmol) was added to a mixture of potassium *tert*-butoxide (1.80 g, 16.0 mmol) in dry THF (40 mL) at 0 °C. The reaction mixture was stirred and heated to 40 °C for 30 min. After cooling to 0 °C *tert*-butyl 2-bromoacetate (3.00 g, 15.4 mmol) was added. The reaction mixture was stirred for 1 h at 0 °C and was then allowed to warm to room temperature. It was stirred for 18 h. The reaction mixture was partitioned between water (100 mL) and EtOAc (100 mL). The organic layer was separated and the aqueous phase was extracted with EtOAc (2 × 100 mL). The combined organic layers were washed with brine (50 mL), dried over Na₂SO₄, filtered and concentrated *in vacuo*. The residue was purified by

silica gel column chromatography using a gradient from petroleum ether/EtOAc (8+1) to petroleum ether/EtOAc (1+1) to yield a colorless oil (2.65 g).

Yield 55%; **¹H NMR** (500 MHz, DMSO-*d*₆) δ 1.40 – 1.43 (m, 9H, C(CH₃)₃), 3.53 – 3.59 (m, 8H), 3.97 – 4.00 (m, 2H), 4.49 (s, 2H, OCH₂), 7.22 – 7.39 (m, 5H, CH_{arom.}); **¹³C NMR** (126 MHz, DMSO-*d*₆) δ 27.7 (C(CH₃)₃), 68.1, 69.1, 69.7, 69.7, 69.8, 72.0 (OCH₂), 80.6 (C(CH₃)₃), 127.3, 127.4, 128.1, 138.4 (C_{arom.}), 169.3 (CO); **LC-MS** (ESI) (90% H₂O to 100% MeOH in 10 min, then 100% MeOH to 20 min, DAD 220-400 nm), *t*_R = 11.01 min, 96% purity, *m/z* [M + NH₄]⁺ calcd for C₁₇H₂₆O₅ 328.21, found 328.0.

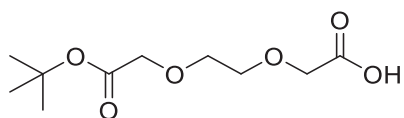
***tert*-Butyl 2-(2-(2-hydroxyethoxy)ethoxy)acetate (**40**)**³²⁷



tert-Butyl 2-(2-(2-(benzyloxy)ethoxy)ethoxy)acetate (**39**, 2.64 g, 8.50 mmol) was dissolved in dry EtOH (20 mL) and was treated with 10% Pd/C (10% m/m) under H₂ (1 atm, balloon) for 18 h. The mixture was filtered through celite and was concentrated *in vacuo* to yield a colorless oil (1.78 g).

Yield 95%; **¹H NMR** (500 MHz, DMSO-*d*₆) δ 1.41 – 1.45 (m, 9H, C(CH₃)₃), 3.40 – 3.45 (m, 2H), 3.46 – 3.51 (m, 2H), 3.51 – 3.55 (m, 2H), 3.56 – 3.59 (m, 2H), 3.97 – 4.01 (m, 2H, OCH₂), 4.52 – 4.58 (m, 1H, OH); **¹³C NMR** (126 MHz, DMSO-*d*₆) δ 27.7 (C(CH₃)₃), 60.2 (CH₂OH), 68.1, 69.6, 69.9, 72.3 (OCH₂), 80.6 (C(CH₃)₃), 169.3 (CO); **LC-MS** (ESI) (90% H₂O to 100% MeOH in 10 min, then 100% MeOH to 20 min), *t*_R = 8.25 min, *m/z* [M + H]⁺ calcd for C₁₀H₂₀O₅ 221.14, found 221.1.

2-(2-(2-(*tert*-Butoxy)-2-oxoethoxy)ethoxy)acetic acid (41**)**

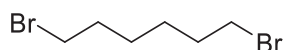


tert-Butyl 2-(2-(2-hydroxyethoxy)ethoxy)acetate (**40**, 1.87 g, 8.50 mmol) was dissolved in MeCN (12.5 mL) and H₂O (12.5 mL). TEMPO (292 mg, 1.87 mmol) was added. (Diacetoxyiodo) benzene (6.02 g, 18.7 mmol) was added portionwise. The reaction mixture was stirred for 16 h at room temperature. The mixture was neutralized by the addition of saturated NaHCO₃ solution (170 mL) and the aqueous layer was washed with EtOAc (2 × 100 mL). The

aqueous phase was acidified with 2 N HCl until $pH \sim 1$. The mixture was extracted with EtOAc (2×100 mL) and the combined organic layers were dried over Na_2SO_4 , filtered and concentrated *in vacuo*. The residue was purified by silica gel column chromatography using a gradient of CH_2Cl_2 to $CH_2Cl_2/MeOH$ (19+1) to yield a colorless oil (1.56 g).

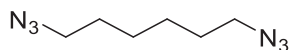
Yield 78%; ^1H NMR (600 MHz, $DMSO-d_6$) δ 1.42 (s, 9H, $C(CH_3)_3$), 3.58 – 3.60 (m, 4H), 3.99 (s, 2H), 4.02 (s, 2H, OCH_2), 12.56 (s, 1H, $COOH$); ^{13}C NMR (151 MHz, $DMSO-d_6$) δ 27.7 ($C(CH_3)_3$), 67.5, 68.1, 69.7, 69.8 (OCH_2), 80.6 ($C(CH_3)_3$), 169.3, 171.5 (CO); LC-MS (ESI) (90% H_2O + 0.1% AcOH to 100% MeCN + 0.1% AcOH in 10 min, then 100% MeCN + 0.1% AcOH to 20 min), t_R = 4.81 min, m/z $[M + H]^+$ calcd for $C_{10}H_{18}O_6$ 235.12, found 235.1.

1,6-Dibromohexane (42)



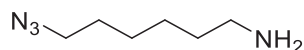
Compound **42** was purchased from Sigma Aldrich/Merck (Darmstadt, Germany).

1,6-Diazidohexane (43)^{328–330}



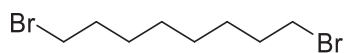
1,6-Dibromohexane (**42**, 1.22 g, 5.00 mmol) was dissolved in dry DMF (10 mL). NaN_3 (1.30 g, 20.0 mmol) was added and the mixture was stirred at 80 °C for 24 h. The reaction mixture was allowed to cool to room temperature and was carefully concentrated. The residue was diluted with H_2O (30 mL) and was extracted with EtOAc (3×30 mL). The combined organic layers were washed with brine (20 mL), dried over Na_2SO_4 , filtered and concentrated *in vacuo* to yield a slightly yellow liquid (819 mg).

Yield 97%; ^1H NMR (500 MHz, $DMSO-d_6$) δ 1.31 – 1.37 (m, 4H), 1.50 – 1.57 (m, 4H, CH_2), 3.32 (t, J = 6.9 Hz, 4H, CH_2N_3); ^{13}C NMR (126 MHz, $DMSO-d_6$) δ 25.6, 28.0 (CH_2), 50.5 (CH_2N_3).

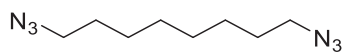
6-Azidohexan-1-amine (44)^{328,329}

1,6-Diazidohexane (**43**, 505 mg, 3.00 mmol) was dissolved in a mixture of THF (10 mL) and H₂O (5 mL). Triphenylphosphine (787 mg, 3.00 mmol) was added and the reaction mixture was stirred at room temperature for 18 h. The reaction was quenched by the addition of 2 N HCl (20 mL). The aqueous layer was separated and extracted with CH₂Cl₂ (3 × 50 mL). The aqueous phase was adjusted to pH~10 by the addition of 2 N NaOH (30 mL) and was extracted with CH₂Cl₂ (3 × 50 mL). The combined organic layers were dried over Na₂SO₄, filtered and concentrated *in vacuo* to yield a colorless liquid (192 mg).

Yield 45%; ¹H NMR (600 MHz, CDCl₃-*d*) δ 1.31 – 1.41 (m, 4H), 1.41 – 1.50 (m, 4H), 1.57 – 1.63 (m, 2H), 2.68 (t, *J* = 7.0 Hz, 2H, CH₂, NH₂), 3.25 (t, *J* = 6.9 Hz, 2H, CH₂N₃); ¹³C NMR (151 MHz, CDCl₃-*d*) δ 26.6, 26.7, 28.9, 33.7 (CH₂), 42.2 (CH₂NH₂), 51.5 (CH₂N₃); LC-MS (ESI) (90% H₂O to 100% MeCN in 10 min, then 100% MeCN to 15 min), *t*_R = 1.99 min, *m/z* [M + H]⁺ calcd for C₆H₁₄N₄ 143.13, found 142.9.

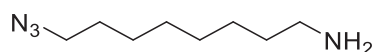
1,8-Dibromooctane (45)

Compound **45** was purchased from Sigma Aldrich/Merck (Darmstadt, Germany).

1,8-Diazidooctane (46)³³¹

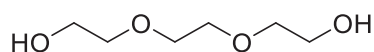
1,8-Dibromooctane (**45**, 2.72 g, 10.0 mmol) was dissolved in dry DMF (10 mL). NaN₃ (2.60 g, 40.0 mmol) was added and the mixture was stirred at 80 °C for 24 h. The reaction mixture was allowed to cool to room temperature and was carefully concentrated. The residue was diluted with H₂O (50 mL) and was extracted with EtOAc (3 × 50 mL). The combined organic layers were washed with brine (20 mL), dried over Na₂SO₄, filtered and concentrated *in vacuo* to yield a colorless liquid (1.60 g).

Yield 82%; ¹H NMR (500 MHz, DMSO-*d*₆) δ 1.26 – 1.35 (m, 8H), 1.48 – 1.57 (m, 4H, CH₂), 3.31 (t, *J* = 6.9 Hz, 4H, CH₂N₃); ¹³C NMR (126 MHz, DMSO-*d*₆) δ 26.0, 28.1, 28.3 (CH₂), 50.6 (CH₂N₃).

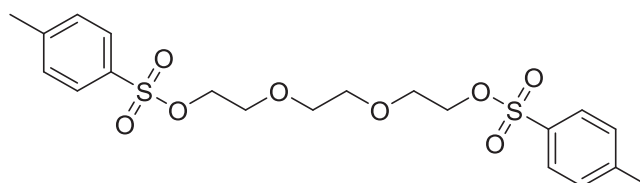
8-Azidoctan-1-amine (47)³³²

1,8-Diazidooctane (**46**, 981 mg, 5.00 mmol) was dissolved in a mixture of THF (10 mL) and H₂O (5 mL). Triphenylphosphine (1.31 g, 5.00 mmol) was added and the reaction mixture was stirred at room temperature for 18 h. The reaction was quenched by the addition of 2 N HCl (20 mL). The aqueous layer was separated and extracted with CH₂Cl₂ (3 × 50 mL). The aqueous phase was adjusted to pH~10 by the addition of 2 N NaOH (30 mL) and was extracted with CH₂Cl₂ (3 × 50 mL). The combined organic layers were dried over Na₂SO₄, filtered and concentrated *in vacuo* to yield a slightly yellow liquid (567 mg).

Yield 67%; ¹H NMR (600 MHz, CDCl₃-*d*) δ 1.27 – 1.39 (m, 10H), 1.40 – 1.44 (m, 2H, CH₂), 1.55 – 1.62 (m, 2H), 2.67 (t, *J* = 7.0 Hz, 2H, CH₂, NH₂), 3.24 (t, *J* = 7.0 Hz, 2H, CH₂N₃); ¹³C NMR (151 MHz, CDCl₃-*d*) δ 26.8, 26.9, 28.9, 29.2, 29.5, 33.9 (CH₂), 42.3 (CH₂NH₂), 51.6 (CH₂N₃); LC-MS (ESI) (90% H₂O to 100% MeCN in 10 min, then 100% MeCN to 15 min), *t*_R = 4.50 min, *m/z* [M + H]⁺ calcd for C₈H₁₈N₄ 171.16, found 171.2.

2,2'-(Ethane-1,2-diylbis(oxy))bis(ethan-1-ol) (48)

Compound **48** was purchased from Sigma Aldrich/Merck (Darmstadt, Germany).

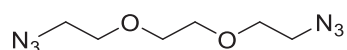
(Ethane-1,2-diylbis(oxy))bis(ethane-2,1-diyl) bis(4-methylbenzenesulfonate) (49)^{286,333,334}

Compound **49** was prepared similar to a previously reported procedure.²⁸⁶ 2,2'-(Ethane-1,2-diylbis(oxy))bis(ethan-1-ol) (**48**, 6.01 g, 40.0 mmol) was dissolved in dry CH₂Cl₂ (40 mL). TEA (10.1 g, 100 mmol) was added. 4-Methylbenzenesulfonyl chloride (16.8 g, 88.0 mmol) was added and the mixture was cooled with a water bath. The reaction mixture was stirred at room temperature for 24 h. The reaction was quenched by the addition of 2 N NaOH (50 mL). The aqueous layer was extracted with CH₂Cl₂ (3 × 50 mL). The combined organic layers were dried over Na₂SO₄, filtered and concentrated *in vacuo*. The crude product was purified by silica

gel column chromatography using petroleum ether/EtOAc (1+1) as eluent to yield a white solid (11.6 g).

Yield 63%; mp: 79-81 °C, lit.³³³ mp: 82-83 °C; ¹H NMR (500 MHz, DMSO-*d*₆) δ 2.41 (s, 6H, CH₃), 3.37 – 3.40 (m, 4H), 3.52 – 3.55 (m, 4H), 4.08 – 4.11 (m, 4H, CH₂), 7.47 (d, *J* = 8.2 Hz, 4H), 7.75 – 7.80 (m, 4H, CH_{arom.}); ¹³C NMR (126 MHz, DMSO-*d*₆) δ 21.0 (CH₃), 67.8, 69.5, 69.9 (CH₂), 127.5, 130.1, 132.4, 144.8 (C_{arom.}); LC-MS (ESI) (90% H₂O to 100% MeCN in 10 min, then 100% MeCN to 15 min, DAD 200-600 nm), *t*_R = 7.38 min, 100% purity, *m/z* [M + NH₄]⁺ calcd for C₂₀H₂₆O₈S₂ 476.14, found 476.2.

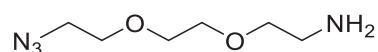
1,2-Bis(2-azidoethoxy)ethane (**50**)^{286,335}



Compound **50** was prepared similar to a previously reported procedure.²⁸⁶ (Ethane-1,2-diylbis(oxy))bis(ethane-2,1-diyl) bis(4-methylbenzenesulfonate) (**49**, 11.5 g, 25.0 mmol) was dissolved in dry DMF (30 mL). NaN₃ (6.50 g, 100 mmol) was added. The reaction mixture was stirred at 80 °C for 24 h. The mixture was allowed to cool to room temperature and was carefully concentrated. The residue was diluted with H₂O (50 mL) and was extracted with EtOAc (3 × 50 mL). The combined organic layers were washed with brine (50 mL), dried over Na₂SO₄, filtered and concentrated *in vacuo* to yield a slightly yellow oil (4.36 g).

Yield 87%; ¹H NMR (500 MHz, DMSO-*d*₆) δ 3.39 (t, *J* = 4.9 Hz, 4H), 3.57 – 3.59 (m, 4H), 3.60 – 3.63 (m, 4H, CH₂); ¹³C NMR (126 MHz, DMSO-*d*₆) δ 50.0 (CH₂N₃), 69.2, 69.7 (CH₂); LC-MS (ESI) (90% H₂O to 100% MeCN in 10 min, then 100% MeCN to 15 min), *t*_R = 4.33 min, *m/z* [M + NH₄]⁺ calcd for C₆H₁₂N₆O₂ 218.14, found 218.1.

2-(2-(2-Azidoethoxy)ethoxy)ethan-1-amine (**51**)^{286,336}

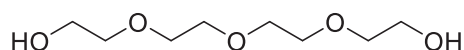


Compound **51** was prepared similar to a previously reported procedure.²⁸⁶ 1,2-Bis(2-azidoethoxy)ethane (**50**, 4.00 g, 20.0 mmol) was dissolved in a mixture of THF (30 mL) and H₂O (10 mL). Triphenylphosphine (5.25 g, 20.0 mmol) was added and the reaction mixture was stirred at room temperature for 18 h. The reaction was quenched by the addition of 2 N HCl (30 mL). The aqueous layer was separated and extracted with CH₂Cl₂ (3 × 50 mL). The aqueous phase was adjusted to pH~10 by the addition of 2 N NaOH (40 mL) and was extracted with

CH₂Cl₂ (3 × 50 mL). The combined organic layers were dried over Na₂SO₄, filtered and concentrated *in vacuo* to yield a slightly yellow oil (1.74 g).

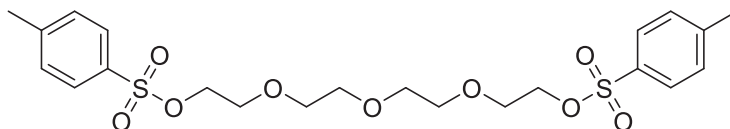
Yield 50%; ¹H NMR (500 MHz, DMSO-*d*₆) δ 2.64 (t, *J* = 5.8 Hz, 2H, CH₂NH₂), 3.34 – 3.41 (m, 4H), 3.50 – 3.54 (m, 2H), 3.55 – 3.59 (m, 2H), 3.59 – 3.62 (m, 2H, CH₂), two protons (NH₂) are missing due to proton exchange; ¹³C NMR (126 MHz, DMSO-*d*₆) δ 41.4 (CH₂NH₂), 50.0 (CH₂N₃), 69.2, 69.6, 69.7, 73.1 (CH₂); LC-MS (ESI) (90% H₂O to 100% MeCN in 10 min, then 100% MeCN to 15 min), *t*_R = 0.75 min, *m/z* [M + H]⁺ calcd for C₆H₁₄N₄O₂ 175.12, found 175.2.

2,2'-((Oxybis(ethane-2,1-diyl))bis(oxy))bis(ethan-1-ol) (52)



Compound **52** was purchased from Sigma Aldrich/Merck (Darmstadt, Germany).

((Oxybis(ethane-2,1-diyl))bis(oxy))bis(ethane-2,1-diyl) bis(4-methylbenzenesulfonate) (53)^{286,337}

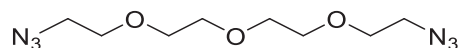


Compound **53** was prepared similar to a previously reported procedure.²⁸⁶ 2,2'-((Oxybis(ethane-2,1-diyl))bis(oxy))bis(ethan-1-ol) (**52**, 7.77 g, 40.0 mmol) was dissolved in dry CH₂Cl₂ (40 mL). TEA (10.1 g, 100 mmol) was added to the solution. 4-Methylbenzenesulfonyl chloride (16.8 g, 88.0 mmol) was added in one portion and the reaction was cooled with a water bath. The reaction mixture was allowed to stir at room temperature for 24 h. The reaction was quenched by the addition of 2 N NaOH (50 mL). The aqueous layer was extracted with CH₂Cl₂ (3 × 50 mL). The combined organic layers were dried over Na₂SO₄, filtered and concentrated *in vacuo*. The crude oil was purified by silica gel column chromatography using petroleum ether/EtOAc (1+1) as eluent to yield a colorless oil (17.1 g).

Yield 85%; ¹H NMR (500 MHz, DMSO-*d*₆) δ 2.42 (s, 6H, CH₃), 3.41 – 3.44 (m, 8H), 3.55 – 3.58 (m, 4H), 4.09 – 4.12 (m, 4H, OCH₂), 7.47 (d, *J* = 8.2 Hz, 4H), 7.78 (d, *J* = 8.1 Hz, 4H, CH_{arom.}); ¹³C NMR (126 MHz, DMSO-*d*₆) δ 21.0 (CH₃), 67.8, 69.6, 69.6, 69.9 (CH₂), 127.5, 130.1, 132.4, 144.8 (C_{arom.}); LC-MS (ESI) (90% H₂O to 100% MeCN in 10 min, then 100%

MeCN to 15 min, DAD 200-600 nm), $t_R = 7.62$ min, 99% purity, m/z $[M + NH_4]^+$ calcd for $C_{22}H_{30}O_9S_2$ 520.17, found 520.3.

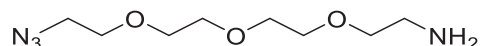
1-Azido-2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethane (54**)**^{286,338}



Compound **54** was prepared similar to a previously reported procedure.²⁸⁶ ((Oxybis(ethane-2,1-diyl))bis(oxy))bis(ethane-2,1-diyl) bis(4-methylbenzenesulfonate) (**53**, 16.59 g, 33.0 mmol) was dissolved in dry DMF (30 mL). NaN_3 (8.58 g, 132 mmol) was added and the mixture was heated to 80 °C for 24 h. The mixture was allowed to cool to room temperature and was carefully concentrated. The residue was diluted with H_2O (50 mL) and extracted with EtOAc (3×50 mL). The combined organic layers were washed with brine (50 mL), dried over Na_2SO_4 , filtered and concentrated *in vacuo* to yield a colorless oil (7.73 g).

Yield 96%; ^1H NMR (500 MHz, $DMSO-d_6$) δ 3.36 – 3.41 (m, 4H), 3.54 – 3.58 (m, 8H), 3.59 – 3.62 (m, 4H, CH_2); ^{13}C NMR (126 MHz, $DMSO-d_6$) δ 50.0 (CH_2N_3), 69.2, 69.7, 69.8 (CH_2); LC-MS (ESI) (90% H_2O to 100% MeCN in 10 min, then 100% MeCN to 15 min), $t_R = 4.41$ min, m/z $[M + NH_4]^+$ calcd for $C_8H_{16}N_6O_3$ 262.16, found 262.1.

2-(2-(2-(2-Azidoethoxy)ethoxy)ethoxy)ethan-1-amine (55**)**^{286,338}

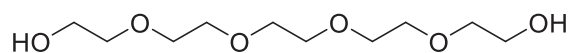


Compound **55** was prepared similar to a previously reported procedure.²⁸⁶ 1-Azido-2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethane (**54**, 7.33 g, 30.0 mmol) was dissolved in a mixture of THF (40 mL) and H_2O (10 mL). Triphenylphosphine (7.87 g, 30.0 mmol) was added and the reaction mixture was stirred at room temperature for 18 h. The reaction was quenched by the addition of 2 N HCl (30 mL). The aqueous layer was separated and extracted with CH_2Cl_2 (3×50 mL). The aqueous phase was adjusted to $pH \sim 10$ by the addition of 2 N NaOH (40 mL) and was extracted with CH_2Cl_2 (3×50 mL). The combined organic layers were dried over Na_2SO_4 , filtered and concentrated *in vacuo* to yield a colorless oil (3.79 g).

Yield 58%; ^1H NMR (500 MHz, $DMSO-d_6$) δ 2.64 (t, $J = 5.8$ Hz, 2H, CH_2NH_2), 3.35 (t, $J = 5.9$ Hz, 2H), 3.39 (t, $J = 4.9$ Hz, 2H), 3.47 – 3.57 (m, 8H), 3.60 (t, $J = 4.9$ Hz, 2H, CH_2), two protons (NH_2) are missing due to proton exchange; ^{13}C NMR (126 MHz, $DMSO-d_6$) δ 41.3 (CH_2NH_2), 50.0 (CH_2N_3), 69.2, 69.5, 69.7, 69.8, 73.1 (CH_2), one signal is missing due to

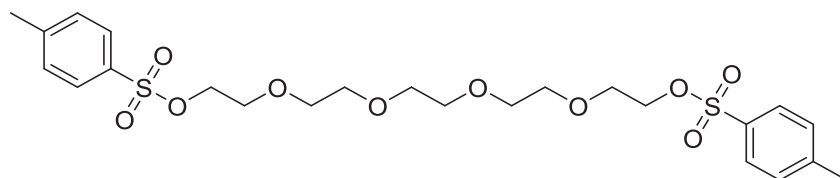
overlapping signals; **LC-MS** (ESI) (90% H₂O to 100% MeCN in 10 min, then 100% MeCN to 15 min), $t_R = 0.96$ min, m/z $[M + H]^+$ calcd for C₈H₁₈N₄O₃ 219.15, found 219.1.

3,6,9,12-Tetraoxatetradecane-1,14-diol (**56**)



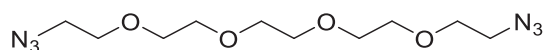
Compound **56** was purchased from TCI (Eschborn, Germany).

3,6,9,12-Tetraoxatetradecane-1,14-diyl bis(4-methylbenzenesulfonate) (**57**)^{286,339}



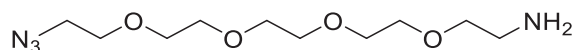
Compound **57** was prepared similar to a previously reported procedure.²⁸⁶ 3,6,9,12-Tetraoxatetradecane-1,14-diol (**56**, 9.53 g, 40.0 mmol) was dissolved in dry CH₂Cl₂ (40 mL). TEA (10.1 g, 100 mmol) was added to the solution. 4-Methylbenzenesulfonyl chloride (16.8 g, 88.0 mmol) was added in one portion and the reaction was cooled with a water bath. The reaction mixture was allowed to stir at room temperature for 24 h. The reaction was quenched by the addition of 2 N NaOH (50 mL). The aqueous layer was extracted with CH₂Cl₂ (3 × 50 mL). The combined organic layers were dried over Na₂SO₄, filtered and concentrated *in vacuo*. The crude oil was purified by silica gel column chromatography using petroleum ether/EtOAc (1+1) as eluent to yield a colorless oil (15.4 g).

Yield 70%; **¹H NMR** (500 MHz, DMSO-*d*₆) δ 2.42 (s, 6H, CH₃), 3.43 – 3.45 (m, 8H), 3.45 – 3.47 (m, 4H), 3.56 – 3.58 (m, 4H), 4.09 – 4.13 (m, 4H, CH₂), 7.47 (d, $J = 8.1$ Hz, 4H), 7.76 – 7.80 (m, 4H, CH_{arom.}); **¹³C NMR** (126 MHz, DMSO-*d*₆) δ 21.0 (CH₃), 67.8, 69.6, 69.7, 69.7, 69.9 (CH₂), 127.5, 130.1, 132.4, 144.8 (C_{arom.}); **LC-MS** (ESI) (90% H₂O to 100% MeCN in 10 min, then 100% MeCN to 15 min, DAD 200-600 nm), $t_R = 7.45$ min, 100% purity, m/z $[M + NH_4]^+$ calcd for C₂₄H₃₄O₁₀S₂ 564.19, found 564.3.

1,14-Diazido-3,6,9,12-tetraoxatetradecane (58)^{286,340}

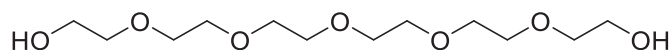
Compound **58** was prepared similar to a previously reported procedure.²⁸⁶ 3,6,9,12-Tetraoxatetradecane-1,14-diyl bis(4-methylbenzenesulfonate) (**57**, 13.7 g, 25.0 mmol) was dissolved in dry DMF (30 mL). NaN₃ (6.50 g, 100 mmol) was added and the mixture was heated to 80 °C for 24 h. The mixture was allowed to cool to room temperature and was carefully concentrated. The residue was diluted with H₂O (50 mL) and extracted with EtOAc (3 × 50 mL). The combined organic layers were washed with brine (50 mL), dried over Na₂SO₄, filtered and concentrated *in vacuo* to yield a colorless oil (5.90 g).

Yield 82%; ¹H NMR (600 MHz, DMSO-*d*₆) δ 3.39 (t, *J* = 4.8 Hz, 4H), 3.53 – 3.57 (m, 12H), 3.59 – 3.61 (m, 4H, CH₂); ¹³C NMR (151 MHz, DMSO-*d*₆) δ 50.0 (CH₂N₃), 69.2, 69.7, 69.8, 69.8 (CH₂); LC-MS (ESI) (90% H₂O to 100% MeCN in 10 min, then 100% MeCN to 15 min), *t*_R = 4.84 min, *m/z* [M + NH₄]⁺ calcd for C₁₀H₂₀N₆O₄ 306.19, found 306.3.

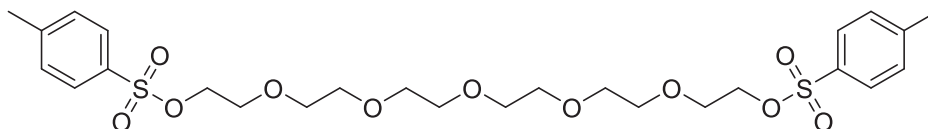
14-Azido-3,6,9,12-tetraoxatetradecan-1-amine (59)^{286,341}

Compound **59** was prepared similar to a previously reported procedure.²⁸⁶ 1,14-Diazido-3,6,9,12-tetraoxatetradecane (**58**, 5.77 g, 20.0 mmol) was dissolved in a mixture of THF (30 mL) and H₂O (10 mL). Triphenylphosphine (5.25 g, 20.0 mmol) was added and the reaction mixture was stirred at room temperature for 18 h. The reaction was quenched by the addition of 2 N HCl (30 mL). The aqueous layer was separated and extracted with CH₂Cl₂ (3 × 50 mL). The aqueous phase was adjusted to pH~10 by the addition of 2 N NaOH (40 mL) and was extracted with CH₂Cl₂ (3 × 50 mL). The combined organic layers were dried over Na₂SO₄, filtered and concentrated *in vacuo* to yield a colorless oil (2.16 g).

Yield 41%; ¹H NMR (500 MHz, DMSO-*d*₆) δ 2.64 (t, *J* = 5.8 Hz, 2H, CH₂NH₂), 3.33 – 3.37 (m, 2H), 3.37 – 3.41 (m, 2H), 3.48 – 3.57 (m, 12H), 3.58 – 3.62 (m, 2H, CH₂), two protons (NH₂) are missing due to proton exchange; ¹³C NMR (126 MHz, DMSO-*d*₆) δ 41.3 (CH₂NH₂), 50.0 (CH₂N₃), 69.2, 69.5, 69.7, 69.8, 69.8, 69.8, 73.1 (CH₂), one signal is missing due to overlapping signals (CH₂); LC-MS (ESI) (90% H₂O to 100% MeCN in 10 min, then 100% MeCN to 15 min), *t*_R = 1.83 min, *m/z* [M + H]⁺ calcd for C₁₀H₂₂N₄O₄ 263.17, found 263.2.

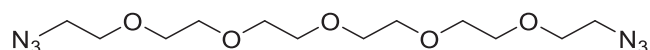
3,6,9,12,15-Pentaoxaheptadecane-1,17-diol (60)

Compound **60** was purchased from TCI (Eschborn, Germany).

3,6,9,12,15-Pentaoxaheptadecane-1,17-diyl bis(4-methylbenzenesulfonate) (61)^{333,342}

3,6,9,12,15-Pentaoxaheptadecane-1,17-diol (**60**, 4.80 g, 17.0 mmol) was dissolved in dry CH_2Cl_2 (40 mL). TEA (4.30 g, 42.5 mmol) was added to the solution. 4-Methylbenzenesulfonyl chloride (7.13 g, 37.4 mmol) was added in one portion and the reaction was cooled with a water bath. The reaction mixture was allowed to stir at room temperature for 24 h. The reaction was quenched by the addition of 2 N NaOH (50 mL). The aqueous layer was extracted with CH_2Cl_2 (3 $\times$ 50 mL). The combined organic layers were dried over Na_2SO_4 , filtered and concentrated *in vacuo*. The crude oil was purified by silica gel column chromatography using a gradient petroleum ether/EtOAc gradient (66% to 100% EtOAc) to yield a colorless oil (8.14 g).

Yield 81%; ^1H NMR (600 MHz, $\text{DMSO}-d_6$) δ 2.42 (s, 6H, CH_3), 3.43 – 3.45 (m, 8H), 3.46 – 3.50 (m, 8H), 3.56 – 3.58 (m, 4H), 4.09 – 4.12 (m, 4H, CH_2), 7.46 – 7.50 (m, 4H), 7.76 – 7.81 (m, 4H, CH_{arom}); ^{13}C NMR (151 MHz, $\text{DMSO}-d_6$) δ 21.0 (CH_3), 67.8, 69.6, 69.7, 69.7, 69.7, 69.9 (CH_2), 127.6, 130.1, 132.4, 144.8 (C_{arom}); LC-MS (ESI) (90% H_2O to 100% MeCN in 10 min, then 100% MeCN to 15 min, DAD 200-600 nm), t_R = 7.45 min, 100% purity, m/z [$\text{M} + \text{NH}_4$] $^+$ calcd for $\text{C}_{26}\text{H}_{38}\text{O}_{11}\text{S}_2$ 608.22, found 608.3.

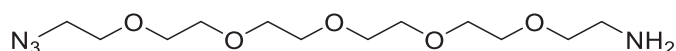
1,17-Diazido-3,6,9,12,15-pentaoxaheptadecane (62)³⁴²

3,6,9,12,15-Pentaoxaheptadecane-1,17-diyl bis(4-methylbenzenesulfonate) (**61**, 7.97 g, 13.5 mmol) was dissolved in dry DMF (20 mL). NaN_3 (3.51 g, 54.0 mmol) was added and the mixture was heated to 80 $^\circ\text{C}$ for 24 h. The mixture was allowed to cool to room temperature and was carefully concentrated. The residue was diluted with H_2O (50 mL) and extracted with

EtOAc (3 × 50 mL). The combined organic layers were washed with brine (50 mL), dried over Na₂SO₄, filtered and concentrated *in vacuo* to yield a colorless oil (4.02 g).

Yield 90%; ¹H NMR (500 MHz, DMSO-*d*₆) δ 3.36 – 3.41 (m, 4H), 3.51 – 3.58 (m, 16H), 3.59 – 3.62 (m, 4H, CH₂); ¹³C NMR (126 MHz, DMSO-*d*₆) δ 50.0 (CH₂N₃), 69.2, 69.7, 69.7, 69.8, 69.8 (CH₂); LC-MS (ESI) (90% H₂O to 100% MeCN in 10 min, then 100% MeCN to 15 min), *t*_R = 4.99 min, *m/z* [M + NH₄]⁺ calcd for C₁₂H₂₄N₆O₅ 350.21, found 350.3.

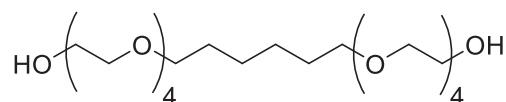
17-Azido-3,6,9,12,15-pentaoxaheptadecan-1-amine (63)³⁴²



1,17-Diazido-3,6,9,12,15-pentaoxaheptadecane (**62**, 3.66 g, 11.0 mmol) was dissolved in a mixture of THF (15 mL) and H₂O (5 mL). Triphenylphosphine (2.89 g, 11.0 mmol) was added and the reaction mixture was stirred at room temperature for 18 h. The reaction was quenched by the addition of 2 N HCl (30 mL). The aqueous layer was separated and extracted with CH₂Cl₂ (3 × 50 mL). The aqueous phase was adjusted to pH~10 by the addition of 2 N NaOH (40 mL) and was extracted with CH₂Cl₂ (3 × 50 mL). The combined organic layers were dried over Na₂SO₄, filtered and concentrated *in vacuo* to yield a colorless oil (1.61 g).

Yield 48%; ¹H NMR (600 MHz, DMSO-*d*₆) δ 2.64 (t, *J* = 5.8 Hz, 2H, CH₂NH₂), 3.35 (t, *J* = 5.8 Hz, 2H), 3.37 – 3.40 (m, 2H), 3.47 – 3.52 (m, 2H), 3.50 – 3.53 (m, 10H), 3.51 – 3.57 (m, 4H), 3.59 – 3.62 (m, 2H, CH₂), two protons (NH₂) are missing due to proton exchange; ¹³C NMR (151 MHz, DMSO-*d*₆) δ 41.3 (CH₂NH₂), 50.0 (CH₂N₃), 69.2, 69.6, 69.7, 69.8, 69.8, 69.8, 69.8, 73.1 (CH₂), two signals (CH₂) are missing due to overlapping signals; LC-MS (ESI) (90% H₂O to 100% MeCN in 10 min, then 100% MeCN to 15 min), *t*_R = 2.91 min, *m/z* [M + H]⁺ calcd for C₁₂H₂₆N₄O₅ 307.20, found 307.3.

3,6,9,12,19,22,25,28-Octaoxatriacontane-1,30-diol (64)

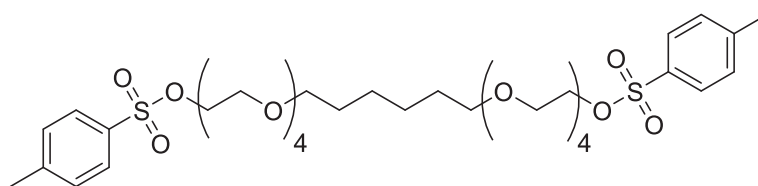


2,2'-((Oxybis(ethane-2,1-diyl))bis(oxy))bis(ethan-1-ol) (**52**, 29.1 g, 150 mmol) was stirred at 70 °C. Sodium (1.72 g, 75.0 mmol) was cut into small pieces and was carefully added. After reaction of the sodium under dissolution, the reaction mixture was heated to 100 °C. 1,6-Dibromohexane (**42**, 7.32 g, 30.0 mmol) was added dropwise and the reaction mixture was

stirred for 3 h at 100 °C. The reaction mixture was allowed to cool to room temperature. H₂O (50 mL) was carefully added and the aqueous layer was extracted with CH₂Cl₂ (3 × 50 mL). The combined organic layers were washed with water (25 mL), dried over Na₂SO₄, filtered and concentrated *in vacuo*. The crude oil was purified by silica gel flash column chromatography using a CH₂Cl₂/MeOH gradient (0% to 10% MeOH) as eluent to yield a slightly yellow oil (5.05 g).

Yield 36%; ¹H NMR (500 MHz, DMSO-*d*₆) δ 1.25 – 1.31 (m, 4H), 1.44 – 1.51 (m, 4H, CH₂), 3.37 (t, *J* = 6.6 Hz, 4H), 3.39 – 3.44 (m, 4H), 3.44 – 3.53 (m, 28H, OCH₂), 4.52 (t, *J* = 5.5 Hz, 2H, OH); ¹³C NMR (126 MHz, DMSO-*d*₆) δ 25.5, 29.1 (CH₂), 60.2 (CH₂OH), 69.4, 69.7, 69.8, 69.8, 70.2, 72.3 (OCH₂), two signals (OCH₂) are missing due to overlapping signals; LC-MS (ESI) (90% H₂O to 100% MeCN in 10 min, then 100% MeCN to 15 min), *t*_R = 4.16 min, *m/z* [M + NH₄]⁺ calcd for C₂₂H₄₆O₁₀ 488.34, found 488.5.

3,6,9,12,19,22,25,28-Octaoxatriacontane-1,30-diyl bis(4-methylbenzenesulfonate) (65)

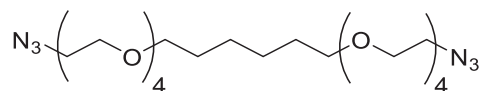


3,6,9,12,19,22,25,28-Octaoxatriacontane-1,30-diol (**64**, 2.35 g, 5.00 mmol) dissolved in dry CH₂Cl₂ (40 mL). TEA (1.26 g, 12.5 mmol) was added to the solution. 4-Methylbenzenesulfonyl chloride (2.10 g, 11.0 mmol) was added in one portion and the reaction was cooled with a water bath. The reaction mixture was allowed to stir at room temperature for 24 h. The reaction was quenched by the addition of 2 N NaOH (50 mL). The aqueous layer was extracted with CH₂Cl₂ (3 × 50 mL). The combined organic layers were dried over Na₂SO₄, filtered and concentrated *in vacuo*. The crude oil was purified by silica gel flash column chromatography using a petroleum ether/EtOAc gradient (0% to 100% EtOAc) to yield a colorless oil (2.24 g).

Yield 58%; ¹H NMR (500 MHz, DMSO-*d*₆) δ 1.23 – 1.31 (m, 4H), 1.42 – 1.50 (m, 4H, CH₂), 2.42 (s, 6H, CH₃), 3.35 (t, *J* = 6.6 Hz, 4H), 3.43 – 3.51 (m, 24H), 3.56 – 3.59 (m, 4H), 4.09 – 4.13 (m, 4H, OCH₂), 7.45 – 7.50 (m, 4H), 7.76 – 7.80 (m, 4H, CH_{arom.}); ¹³C NMR (126 MHz, DMSO-*d*₆) δ 21.0 (CH₃), 25.4, 29.1 (CH₂), 67.8, 69.4, 69.6, 69.7, 69.7, 69.9, 70.2 (OCH₂), 127.5, 130.1, 132.4, 144.8 (C_{arom.}), two signals (OCH₂) are missing due to overlapping signals;

LC-MS (ESI) (90% H₂O to 100% MeCN in 10 min, then 100% MeCN to 15 min, DAD 200-600 nm), t_R = 8.28 min, 98% purity, m/z [M + NH₄]⁺ calcd for C₃₆H₅₈O₁₄S₂ 796.36, found 796.6.

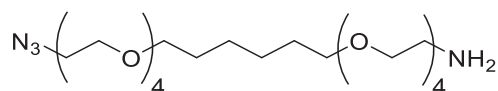
1,30-Diazido-3,6,9,12,19,22,25,28-octaoxatriacontane (66)



3,6,9,12,19,22,25,28-Octaoxatriacontane-1,30-diyl bis(4-methylbenzenesulfonate) (**65**, 1.95 g, 2.50 mmol) was dissolved in dry DMF (10 mL). NaN₃ (650 mg, 10.0 mmol) was added. The mixture was stirred at 80 °C for 24 h. The mixture was allowed to cool to room temperature and was carefully concentrated. The residue was diluted with H₂O (50 mL) and was extracted with EtOAc (3 × 50 mL). The combined organic layers were washed with water (50 mL), dried over Na₂SO₄, filtered and concentrated *in vacuo* to yield a colorless oil (1.16 g).

Yield 89%; ¹H NMR (600 MHz, DMSO-*d*₆) δ 1.26 – 1.32 (m, 4H), 1.45 – 1.51 (m, 4H, CH₂), 3.34 – 3.41 (m, 8H), 3.44 – 3.48 (m, 4H), 3.49 – 3.57 (m, 20H), 3.58 – 3.62 (m, 4H, OCH₂, CH₂N₃); ¹³C NMR (151 MHz, DMSO-*d*₆) δ 25.5, 29.2 (CH₂), 50.0 (CH₂N₃), 69.2, 69.5, 69.7, 69.8, 69.8, 70.2 (OCH₂), two signals (OCH₂) are missing due to overlapping signals; **LC-MS** (ESI) (90% H₂O to 100% MeCN in 10 min, then 100% MeCN to 15 min), t_R = 6.80 min, m/z [M + NH₄]⁺ calcd for C₂₂H₄₄N₆O₈ 538.36, found 538.6.

30-Azido-3,6,9,12,19,22,25,28-octaoxatriacontan-1-amine (67)

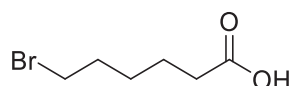


1,30-Diazido-3,6,9,12,19,22,25,28-octaoxatriacontane (**66**, 1.04 g, 2.00 mmol) was dissolved in a mixture of THF (10 mL) and H₂O (5 mL). Triphenylphosphine (525 mg, 2.00 mmol) was added and the reaction mixture was stirred at room temperature for 18 h. The reaction was quenched by the addition of 2 N HCl (15 mL). The aqueous layer was separated and extracted with CH₂Cl₂ (3 × 25 mL). The aqueous phase was adjusted to pH~10 by the addition of 2 N NaOH (20 mL) and was extracted with CH₂Cl₂ (3 × 25 mL). The combined organic layers were dried over Na₂SO₄, filtered and concentrated *in vacuo* to yield a colorless oil (641 mg).

Yield 65%; ¹H NMR (600 MHz, DMSO-*d*₆) δ 1.26 – 1.32 (m, 4H), 1.45 – 1.50 (m, 4H, CH₂), 2.64 (t, *J* = 5.8 Hz, 2H, CH₂NH₂), 3.32 – 3.41 (m, 8H), 3.44 – 3.48 (m, 4H), 3.48 – 3.57 (m,

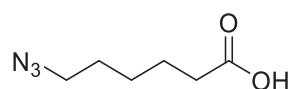
20H), 3.58 – 3.63 (m, 2H, OCH₂, CH₂N₃), two protons (NH₂) are missing due to proton exchange; ¹³C NMR (151 MHz, DMSO-*d*₆) δ 25.5, 29.2 (CH₂), 41.3 (CH₂NH₂), 50.0 (CH₂N₃), 69.2, 69.5, 69.6, 69.7, 69.8, 69.8, 69.8, 69.8, 70.2, 73.1 (OCH₂); LC-MS (ESI) (90% H₂O to 100% MeCN in 10 min, then 100% MeCN to 15 min), *t*_R = 5.26 min, *m/z* [M + H]⁺ calcd for C₂₂H₄₆N₄O₈ 495.34, found 495.4.

6-Bromohexanoic acid (68)



Compound **68** was purchased from Oxchem (Irwindale, United States of America).

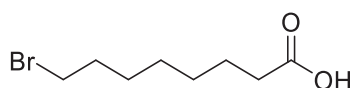
6-Azidohexanoic acid (69)²⁸⁸



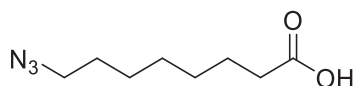
Compound **69** was prepared similar to a previously reported procedure.²⁸⁸ 6-Bromohexanoic acid (**68**, 975 mg, 5.00 mmol) was dissolved in dry DMF (12 mL). NaN₃ (390 mg, 6.00 mmol) was added. The reaction mixture was stirred for 24 h at 80 °C. The reaction was quenched by the addition of water (30 mL). The aqueous phase was extracted with Et₂O (3 × 50 mL). The combined organic layers were washed with brine (50 mL), dried over Na₂SO₄, filtered and concentrated *in vacuo* to yield a colorless oil (724 mg).

Yield 92%; ¹H NMR (600 MHz, DMSO-*d*₆) δ 1.29 – 1.36 (m, 2H), 1.48 – 1.55 (m, 4H), 2.21 (t, *J* = 7.3 Hz, 2H, CH₂), 3.31 (t, *J* = 6.9 Hz, 2H, CH₂N₃), 11.97 (s, 1H, COOH); ¹³C NMR (151 MHz, DMSO-*d*₆) δ 24.0, 25.7, 28.0, 33.5 (CH₂), 50.5 (CH₂N₃), 174.3 (COOH); LC-MS (ESI) (90% H₂O + 0.1% HCOOH to 100% MeCN + 0.1% HCOOH in 10 min), *t*_R = 3.91 min, *m/z* [M - H]⁻ calcd for C₆H₁₁N₃O₂ 156.08, found 156.1.

8-Bromooctanoic acid (70)

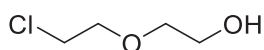


Compound **70** was purchased from TCI (Eschborn, Germany).

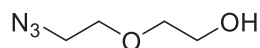
8-Azidooctanoic acid (71)²⁸⁸

Compound **71** was prepared similar to a previously reported procedure.²⁸⁸ 8-Bromooctanoic acid (**70**, 1.12 g, 5.00 mmol) was dissolved in dry DMF (12 mL). NaN₃ (390 mg, 6.00 mmol) was added. The reaction mixture was stirred at 80 °C for 24 h. The reaction mixture was allowed to cool to room temperature and was quenched by the addition of water (30 mL). The aqueous phase was extracted with Et₂O (3 × 50 mL). The combined organic layers were washed with brine (50 mL), dried over Na₂SO₄, filtered and concentrated *in vacuo* to yield a slightly yellow liquid (833 mg).

Yield 90%; ¹H NMR (500 MHz, DMSO-*d*₆) δ 1.23 – 1.35 (m, 6H), 1.45 – 1.56 (m, 4H), 2.16 – 2.22 (m, 2H, CH₂), 11.92 (s, 1H, COOH), one signal (2H, CH₂N₃) is obscured by the solvent signal; ¹³C NMR (126 MHz, DMSO-*d*₆) δ 24.3, 25.9, 28.1, 28.2, 28.3, 33.6 (CH₂), 50.6 (CH₂N₃), 174.4 (COOH); LC-MS (ESI) (90% H₂O + 0.1% HCOOH to 100% MeCN + 0.1% HCOOH in 10 min, then 100% MeCN + 0.1% HCOOH to 15 min), *t*_R = 7.00 min, *m/z* [M - H]⁻ calcd for C₈H₁₅N₃O₂ 184.11, 184.0.

2-(2-Chloroethoxy)ethan-1-ol (72)

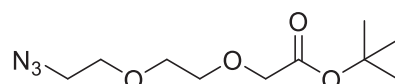
Compound **72** was purchased from Sigma Aldrich/Merck (Darmstadt, Germany).

2-(2-Azidoethoxy)ethan-1-ol (73)³⁴³

NaN₃ (1.15 g, 17.7 mmol) was dissolved in DMF (30 mL). 2-(2-Chloroethoxy)ethan-1-ol (**72**, 1.42 g, 11.4 mmol) was added and the reaction mixture was stirred at 100 °C under for 48 h. The reaction mixture was diluted with 5% LiCl solution (30 mL) and was extracted with EtOAc (6 × 75 mL). The organic layers were washed with brine (50 mL), dried over Na₂SO₄, filtered and concentrated *in vacuo*. The residue was purified by silica gel column chromatography using petroleum ether/EtOAc (1+2) as eluent to yield a colorless oil (1.14 g).

Yield 77%; $^1\text{H NMR}$ (600 MHz, $\text{DMSO-}d_6$) δ 3.38 – 3.40 (m, 2H), 3.45 – 3.47 (m, 2H), 3.49 – 3.52 (m, 2H), 3.59 – 3.61 (m, 2H, CH_2), one signal (1H, OH) is missing due to proton exchange; $^{13}\text{C NMR}$ (151 MHz, $\text{DMSO-}d_6$) δ 50.0 (CH_2N_3), 60.2 (CH_2OH), 69.2, 72.1 (OCH_2); **LC-MS** (ESI) (90% H_2O to 100% MeOH in 10 min, then 100% MeOH to 20 min), t_{R} = 1.79 min, m/z $[\text{M} + \text{H}]^+$ calcd for $\text{C}_4\text{H}_9\text{N}_3\text{O}_2$ 132.08, found 132.0.

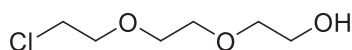
***tert*-Butyl 2-(2-(2-azidoethoxy)ethoxy)acetate (**74**)**³⁴⁴



2-(2-Azidoethoxy)ethan-1-ol (**73**, 1.13 g, 8.60 mmol) was dissolved in toluene (20 mL) and cooled to 0 °C. TBAHS (1.46 g, 4.30 mmol) and *tert*-butyl bromoacetate (5.03 g, 25.8 mmol) were added. Aqueous 9.5 M NaOH (2.7 mL) was added. The reaction mixture was stirred for 22 h at room temperature. The mixture was diluted with H_2O (100 mL) and extracted with Et_2O (3×50 mL). The combined organic layers were washed with brine (50 mL), dried over Na_2SO_4 , filtered and concentrated *in vacuo*. The residue was purified by silica gel column chromatography using petroleum ether/ EtOAc (3+1) as eluent to yield a colorless oil (907 mg).

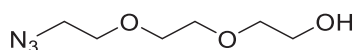
Yield 43%; $^1\text{H NMR}$ (500 MHz, $\text{DMSO-}d_6$) δ 1.42 (s, 9H, $\text{C}(\text{CH}_3)_3$), 3.38 – 3.41 (m, 2H), 3.57 – 3.62 (m, 6H, CH_2N_3 , OCH_2), 3.99 (s, 2H, CH_2COO); $^{13}\text{C NMR}$ (126 MHz, $\text{DMSO-}d_6$) δ 27.7 ($\text{C}(\text{CH}_3)_3$), 50.0 (CH_2N_3), 68.1, 69.2, 69.6, 69.8 (OCH_2), 80.6 ($\text{C}(\text{CH}_3)_3$), 169.3 (COO); **LC-MS** (ESI) (90% H_2O to 100% MeOH in 10 min, then 100% MeOH to 20 min), t_{R} = 10.12 min, m/z $[\text{M} + \text{NH}_4]^+$ calcd for $\text{C}_{10}\text{H}_{19}\text{N}_3\text{O}_4$ 263.17, found 262.9.

2-(2-(2-Chloroethoxy)ethoxy)ethan-1-ol (75**)**



Compound **75** was purchased from TCI (Eschborn, Germany).

2-(2-(2-Azidoethoxy)ethoxy)ethan-1-ol (76**)**²⁸⁹

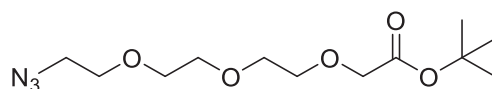


Compound **76** was prepared similar to a previously reported procedure.²⁸⁹ NaN_3 (1.15 g, 17.7 mmol) was dissolved in dry DMF (30 mL). 2-(2-(2-Chloroethoxy)ethoxy)ethan-1-ol (**75**,

1.92 g, 11.4 mmol) was added and the reaction mixture was stirred at 100 °C under argon for 48 h. The reaction mixture was diluted with 5% LiCl solution (30 mL) and was extracted with EtOAc (6 × 75 mL). The organic layers were washed with brine (50 mL), dried over Na₂SO₄, filtered and concentrated *in vacuo*. The residue was purified by silica gel column chromatography using petroleum ether/EtOAc (1+2) as eluent to yield a colorless oil (1.13 g).

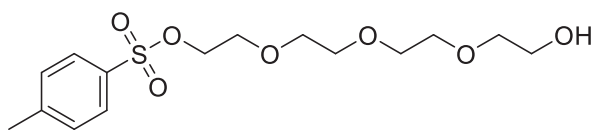
Yield 57%; ¹H NMR (500 MHz, DMSO-*d*₆) δ 3.37 – 3.41 (m, 2H), 3.41 – 3.45 (m, 2H), 3.47 – 3.51 (m, 2H), 3.52 – 3.55 (m, 2H), 3.55 – 3.58 (m, 2H), 3.57 – 3.63 (m, 2H, CH₂N₃, OCH₂), 4.53 (t, *J* = 5.4 Hz, 1H, OH); ¹³C NMR (126 MHz, DMSO-*d*₆) δ 50.0 (CH₂N₃), 60.2 (CH₂OH), 69.2, 69.7, 69.7, 72.3 (OCH₂); LC-MS (ESI) (90% H₂O to 100% MeOH in 10 min, then 100% MeOH to 20 min), *t*_R = 4.19 min, *m/z* [M + H]⁺ calcd for C₆H₁₃N₃O₃ 176.10, found 176.1.

***tert*-Butyl 2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)acetate (77)**²⁸⁹



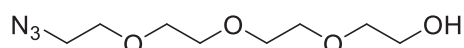
Compound **77** was prepared similar to a previously reported procedure.²⁸⁹ 2-(2-(2-Azidoethoxy)ethoxy)ethan-1-ol (**76**, 1.05 g, 6.00 mmol) was dissolved in toluene (20 mL) and cooled to 0 °C. TBAHS (1.02 g, 3.00 mmol) and *tert*-butyl bromoacetate (3.51 g, 18.0 mmol) were added. An aqueous 9.5 M NaOH solution (2 mL) was added. The reaction mixture was stirred at room temperature for 22 h at room temperature. The mixture was diluted with water (100 mL) and extracted with Et₂O (3 × 50 mL). The combined organic layers were washed with brine (50 mL), dried over Na₂SO₄, filtered and concentrated *in vacuo*. The residue was purified by silica gel column chromatography using petroleum ether/EtOAc (2+1) as eluent to yield a colorless oil (750 mg).

Yield 43%; ¹H NMR (600 MHz, DMSO-*d*₆) δ 1.42 (s, 9H, C(CH₃)₃), 3.37 – 3.40 (m, 2H), 3.53 – 3.58 (m, 8H), 3.59 – 3.61 (m, 2H, CH₂N₃, OCH₂), 3.98 (s, 2H, CH₂COO); ¹³C NMR (151 MHz, DMSO-*d*₆) δ 27.7 (C(CH₃)₃), 50.0 (CH₂N₃), 68.1, 69.2, 69.7, 69.7, 69.8 (OCH₂), 80.6 (C(CH₃)₃), 169.3 (COO), one signal (OCH₂) is missing due to overlapping signals; LC-MS (ESI) (90% H₂O to 100% MeOH in 10 min, then 100% MeOH to 20 min), *t*_R = 10.18 min, *m/z* [M + NH₄]⁺ calcd for C₁₂H₂₃N₃O₅ 307.20, found 307.2.

2-(2-(2-(2-Hydroxyethoxy)ethoxy)ethoxy)ethyl 4-methylbenzenesulfonate (78)²⁹⁰

2,2'-((Oxybis(ethane-2,1-diyl))bis(oxy))bis(ethan-1-ol) (**52**, 7.77 g, 40.0 mmol) was dissolved in dry CH₂Cl₂ (30 mL) and cooled to 0 °C. TEA (3.04 g, 30.0 mmol) and 4-methylbenzenesulfonyl chloride (3.81 g, 20.0 mmol) were added at 0 °C. The reaction mixture was stirred at room temperature for 18 h. The reaction was quenched by the addition of water (50 mL). The organic layer was separated and washed with water (3 × 50 mL). The organic layers were dried over Na₂SO₄, filtered and concentrated *in vacuo*. The crude oil was purified by silica gel flash column chromatography using a CH₂Cl₂/MeOH gradient (0% to 5% MeOH) as eluent to yield a colorless oil (2.79 g).

Yield 40%; ¹H NMR (600 MHz, DMSO-*d*₆) δ 2.42 (s, 3H, CH₃), 3.39 – 3.42 (m, 2H), 3.45 (s, 4H), 3.46 – 3.50 (m, 6H), 3.56 – 3.59 (m, 2H), 4.10 – 4.13 (m, 2H, OCH₂), 4.54 (t, *J* = 5.5 Hz, 1H, OH), 7.47 – 7.50 (m, 2H), 7.77 – 7.80 (m, 2H, CH_{arom.}); ¹³C NMR (151 MHz, DMSO-*d*₆) δ 21.1 (CH₃), 60.2 (CH₂OH), 67.9, 69.6, 69.7, 69.7, 69.8, 69.9, 72.3 (OCH₂), 127.6, 130.1, 132.4, 144.9 (C_{arom.}); LC-MS (ESI) (90% H₂O to 100% MeCN in 10 min, then 100% MeCN to 15 min, DAD 200-600 nm), *t*_R = 4.83 min, 99% purity, *m/z* [M + NH₄]⁺ calcd for C₁₅H₂₄O₇S 366.16, found 366.2.

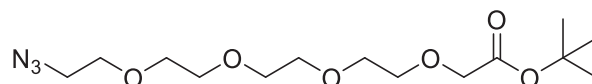
2-(2-(2-(2-Azidoethoxy)ethoxy)ethoxy)ethan-1-ol (79)³⁴⁵

2-(2-(2-(2-Hydroxyethoxy)ethoxy)ethoxy)ethyl 4-methylbenzenesulfonate (**78**, 1.74 g, 5.00 mmol) was dissolved in dry DMF (10 mL). NaN₃ (650 mg, 10.0 mmol) was added and the mixture was stirred at 80 °C for 24 h. The mixture was allowed to cool to room temperature and was carefully concentrated. The residue was diluted with H₂O (50 mL) and extracted with CH₂Cl₂ (3 × 50 mL). The combined organic layers were washed with H₂O (20 mL), dried over Na₂SO₄, filtered and concentrated *in vacuo*. The crude oil was purified by silica gel column chromatography using CH₂Cl₂/MeOH (19+1) as eluent to yield a colorless oil (1.08 g).

Yield 98%; ¹H NMR (600 MHz, DMSO-*d*₆) δ 3.38 – 3.40 (m, 2H), 3.40 – 3.43 (m, 2H), 3.47 – 3.50 (m, 2H), 3.51 – 3.57 (m, 8H), 3.59 – 3.61 (m, 2H, CH₂N₃, OCH₂), 4.54 (t, *J* = 5.5 Hz, 1H, OH); ¹³C NMR (151 MHz, DMSO-*d*₆) δ 50.0 (CH₂N₃), 60.2 (CH₂OH), 69.2, 69.7, 69.7,

69.8, 69.8, 72.3 (OCH₂); **LC-MS** (ESI) (90% H₂O to 100% MeCN in 10 min, then 100% MeCN to 15 min), t_R = 1.72 min, m/z [M + NH₄]⁺ calcd for C₈H₁₇N₃O₄ 237.16, found 237.2.

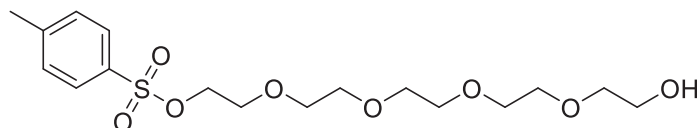
***tert*-Butyl 14-azido-3,6,9,12-tetraoxatetradecanoate (80)**²⁶⁵



2-(2-(2-(2-Azidoethoxy)ethoxy)ethoxy)ethan-1-ol (**79**, 877 mg, 4.00 mmol) was dissolved in dry toluene (20 mL) and cooled to 0 °C. TBAHS (679 mg, 2.00 mmol) and *tert*-butyl bromoacetate (2.34 g, 12.0 mmol) were added. Aqueous 9.5 M NaOH solution (1.26 mL) was added. The reaction mixture was stirred for 22 h at room temperature. The mixture was diluted with H₂O (100 mL) and was extracted with Et₂O (3 × 50 mL). The combined organic layers were washed with brine (50 mL), dried over Na₂SO₄, filtered and concentrated *in vacuo*. The crude oil was purified by silica gel column chromatography using CH₂Cl₂/MeOH (29+1) as eluent to yield a colorless oil (338 mg).

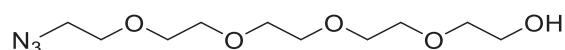
Yield 25%; ¹H NMR (600 MHz, DMSO-*d*₆) δ 1.42 (s, 9H, C(CH₃)₃), 3.37 – 3.40 (m, 2H), 3.51 – 3.58 (m, 12H), 3.59 – 3.61 (m, 2H, CH₂N₃, OCH₂), 3.98 (s, 2H, CH₂COO); ¹³C NMR (151 MHz, DMSO-*d*₆) δ 27.7 (C(CH₃)₃), 50.0 (CH₂N₃), 68.1, 69.2, 69.7, 69.7, 69.7, 69.8, 69.8, 69.8 (OCH₂), 80.6 (C(CH₃)₃), 169.3 (COO); **LC-MS** (ESI) (90% H₂O to 100% MeCN in 10 min, then 100% MeCN to 15 min), t_R = 5.97 min, m/z [M + NH₄]⁺ calcd for C₁₄H₂₇N₃O₆ 351.22, found 351.4.

14-Hydroxy-3,6,9,12-tetraoxatetradecyl 4-methylbenzenesulfonate (81)³⁴⁶

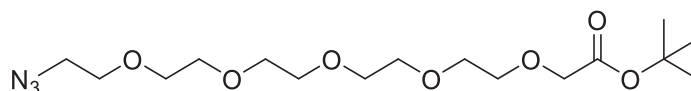


3,6,9,12-Tetraoxatetradecane-1,14-diol (**56**, 9.53 g, 40.0 mmol) was dissolved in dry CH₂Cl₂ (30 mL) and cooled to 0 °C. TEA (3.04 g, 30.0 mmol) and 4-methylbenzenesulfonyl chloride (3.81 g, 20.0 mmol) were added at 0 °C. The reaction mixture was stirred at room temperature for 18 h. The reaction was quenched by the addition of water (50 mL). The organic layer was separated and washed with water (3 × 50 mL). The combined organic layers were dried over Na₂SO₄, filtered and concentrated *in vacuo*. The crude oil was purified by silica gel flash

14-Azido-3,6,9,12-tetraoxatetradecan-1-ol (82)³⁴⁰

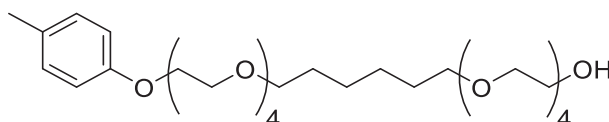


Yield 90%; **¹H NMR** (600 MHz, DMSO-*d*₆) δ 3.36 – 3.41 (m, 2H), 3.40 – 3.43 (m, 2H), 3.47 – 3.50 (m, 2H), 3.50 – 3.53 (m, 8H), 3.53 – 3.57 (m, 4H), 3.59 – 3.61 (m, 2H, CH₂N₃, OCH₂), 4.54 (s, 1H, OH); **¹³C NMR** (151 MHz, DMSO-*d*₆) δ 50.0 (CH₂N₃), 60.2 (CH₂OH), 69.2, 69.7, 69.8, 69.8, 69.8, 72.3 (OCH₂), two signals (OCH₂) are missing due to overlapping signals; **LC-MS** (ESI) (90% H₂O to 100% MeCN in 10 min, then 100% MeCN to 15 min), *t*_R = 2.66 min, *m/z* [M + NH₄]⁺ calcd for C₁₀H₂₁N₃O₅ 281.18, found 281.3.

***tert*-Butyl 17-azido-3,6,9,12,15-pentaoxaheptadecanoate (83)**³⁴⁷

14-Azido-3,6,9,12-tetraoxatetradecan-1-ol (**82**, 1.05 g, 4.00 mmol) was dissolved in dry toluene (20 mL) and cooled to 0 °C. TBAHS (679 mg, 2.00 mmol) and *tert*-butyl bromoacetate (2.34 g, 12.0 mmol) were added. Aqueous 9.5 M NaOH solution (1.26 mL) was added. The reaction mixture was stirred for 22 h at room temperature. The mixture was diluted with H₂O (100 mL) and was extracted with Et₂O (3 × 50 mL). The combined organic layers were washed with brine (50 mL), dried over Na₂SO₄, filtered and concentrated *in vacuo*. The crude oil was purified by silica gel column chromatography using CH₂Cl₂/MeOH (29+1) as eluent to yield a colorless oil (462 mg).

Yield 31%; ¹H NMR (600 MHz, DMSO-*d*₆) δ 1.42 (s, 9H, C(CH₃)₃), 3.37 – 3.41 (m, 2H), 3.51 – 3.58 (m, 16H), 3.59 – 3.62 (m, 2H, CH₂N₃, OCH₂), 3.98 (s, 2H, CH₂COO); ¹³C NMR (151 MHz, DMSO-*d*₆) δ 27.7 (C(CH₃)₃), 50.0 (CH₂N₃), 68.1, 69.2, 69.7, 69.7, 69.8, 69.8, 69.8, 69.8 (OCH₂), 80.6 (C(CH₃)₃), 169.3 (COO), two signals (OCH₂) are missing due to overlapping signals; LC-MS (ESI) (90% H₂O to 100% MeCN in 10 min, then 100% MeCN to 15 min), *t*_R = 5.96 min, *m/z* [M + NH₄]⁺ calcd for C₁₆H₃₁N₃O₇ 395.25, found 395.4.

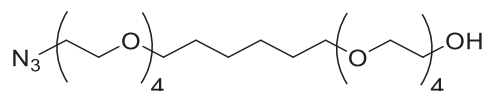
30-Hydroxy-3,6,9,12,19,22,25,28-octaoxatriacontyl 4-methylbenzenesulfonate (84)

3,6,9,12,19,22,25,28-Octaoxatriacontane-1,30-diol (**64**, 4.71 g, 10.0 mmol) was dissolved in dry CH₂Cl₂ (30 mL) and cooled to 0 °C. TEA (607 mg, 6.00 mmol) and 4-methylbenzenesulfonyl chloride (763 mg, 4.00 mmol) were added at 0 °C. The reaction mixture was stirred at room temperature for 18 h. The reaction was quenched by the addition of water (50 mL). The organic layer was separated and washed with water (3 × 50 mL). The organic layer was dried over Na₂SO₄, filtered and concentrated *in vacuo*. The crude oil was purified by silica gel flash column chromatography using an EtOAc/EtOH gradient (0% to 3% EtOH) as eluent to yield a slightly yellow oil (1.26 g).

Yield 50%; ¹H NMR (600 MHz, DMSO-*d*₆) δ 1.25 – 1.30 (m, 4H), 1.44 – 1.50 (m, 4H, CH₂), 2.42 (s, 3H, CH₃), 3.34 – 3.37 (m, 4H), 3.40 – 3.42 (m, 2H), 3.43 – 3.52 (m, 26H), 3.55 – 3.60

(m, 2H), 4.08 – 4.14 (m, 2H, OCH₂), 4.54 (t, J = 5.5 Hz, 1H, OH), 7.48 (d, J = 8.0 Hz, 2H), 7.78 (d, J = 8.3 Hz, 2H, CH_{arom.}); ¹³C NMR (151 MHz, DMSO-*d*₆) δ 21.0 (CH₃), 25.5, 29.2 (CH₂), 60.2 (CH₂OH), 67.9, 69.4, 69.6, 69.7, 69.8, 69.8, 69.9, 70.2, 72.3 (OCH₂), 127.6, 130.1, 132.4, 144.8 (C_{arom.}); LC-MS (ESI) (90% H₂O to 100% MeCN in 10 min, then 100% MeCN to 15 min, DAD 200-600 nm), t_R = 6.29 min, 94% purity, m/z [M + NH₄]⁺ calcd for C₂₉H₅₂O₁₂S 642.35, found 642.7.

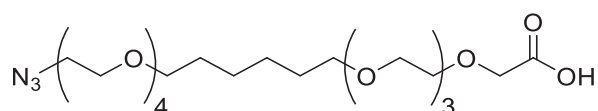
30-Azido-3,6,9,12,19,22,25,28-octaoxatriacontan-1-ol (85)



30-Hydroxy-3,6,9,12,19,22,25,28-octaoxatriacontyl 4-methylbenzenesulfonate (**84**, 1.25 g, 2.00 mmol) was dissolved in dry DMF (10 mL). NaN₃ (260 mg, 4.00 mmol) was added and the mixture was stirred at 80 °C for 24 h. The mixture was allowed to cool to room temperature and was carefully concentrated. The residue was diluted with H₂O (50 mL) and extracted with EtOAc (3 × 50 mL). The combined organic layers were washed with brine (20 mL), dried over Na₂SO₄, filtered and concentrated *in vacuo* to yield a colorless oil (892 mg).

Yield 90%; ¹H NMR (600 MHz, DMSO-*d*₆) δ 1.25 – 1.32 (m, 4H), 1.45 – 1.51 (m, 4H, CH₂), 3.34 – 3.43 (m, 8H), 3.44 – 3.57 (m, 26H), 3.59 – 3.62 (m, 2H, CH₂N₃, OCH₂), 4.54 (t, J = 5.5 Hz, 1H, OH); ¹³C NMR (151 MHz, DMSO-*d*₆) δ 25.5, 29.2 (CH₂), 50.0 (CH₂N₃), 60.2 (CH₂OH), 69.2, 69.5, 69.7, 69.8, 69.8, 69.8, 69.8, 70.2, 72.3 (OCH₂); LC-MS (ESI) (90% H₂O to 100% MeCN in 10 min, then 100% MeCN to 15 min), t_R = 5.53 min, m/z [M + NH₄]⁺ calcd for C₂₂H₄₅N₃O₉ 513.35, found 513.5.

30-Azido-3,6,9,12,19,22,25,28-octaoxatriacontanoic acid (86)

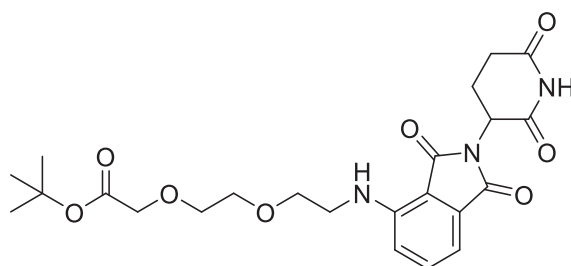


30-Azido-3,6,9,12,19,22,25,28-octaoxatriacontan-1-ol (**85**, 843 mg, 1.70 mmol) was dissolved in MeCN (6 mL) and H₂O (6 mL). TEMPO (58 mg, 370 μmol) was added. BAIB (1.20 g, 3.74 mmol) was added portionwise. The mixture was stirred at room temperature for 16 h. The mixture was neutralized by the addition of saturated NaHCO₃ solution (85 mL) and the aqueous layer was washed with EtOAc (2 × 50 mL). The aqueous phase was acidified with 2 N HCl

to pH~1. The mixture was extracted with EtOAc (2 × 50 mL) and the combined organic layers were dried over Na₂SO₄, filtered and concentrated *in vacuo*. The residue was purified by silica gel column chromatography using a gradient of CH₂Cl₂ to CH₂Cl₂/MeOH (9+1) to yield a colorless oil (561 mg).

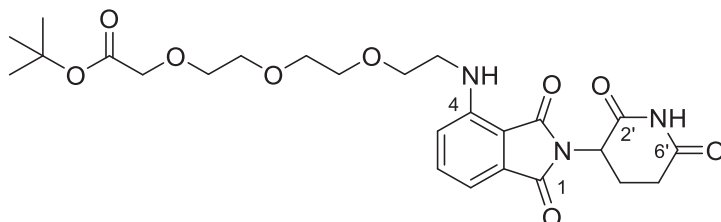
Yield 65%; ¹H NMR (600 MHz, DMSO-*d*₆) δ 1.26 – 1.32 (m, 4H), 1.45 – 1.51 (m, 4H, CH₂), 3.35 – 3.40 (m, 6H), 3.45 – 3.48 (m, 4H), 3.49 – 3.58 (m, 20H), 3.59 – 3.62 (m, 2H, OCH₂), 3.98 (s, 2H, CH₂COO), one proton (COOH) is missing due to proton exchange; ¹³C NMR (151 MHz, DMSO-*d*₆) δ 25.5, 29.2 (CH₂), 50.0 (CH₂N₃), 67.8, 69.2, 69.5, 69.7, 69.7, 69.8, 69.8, 70.2 (OCH₂), 171.7 (COOH); LC-MS (ESI) (90% H₂O + 0.1% HCOOH to 100% MeCN + 0.1% HCOOH in 10 min, then 100% MeCN + 0.1% HCOOH to 15 min), *t*_R = 5.44 min, *m/z* [M - H]⁻ calcd for C₂₂H₄₃N₃O₁₀ 508.28, found 508.4.

***tert*-Butyl 2-(2-(2-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisindolin-4-yl)amino)ethoxy)ethoxy)acetate (87)**²⁴⁹



Compound **87** was prepared as described before.³²³

***tert*-Butyl 2-(2-(2-(2-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisindolin-4-yl)amino)ethoxy)ethoxy)ethoxy)acetate (88)**²⁴⁹

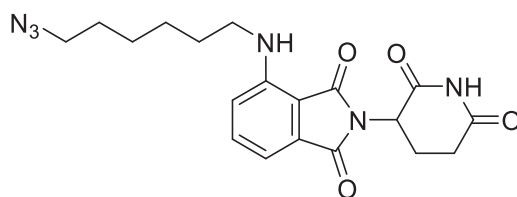


Compound **88** was prepared similar to a previously reported procedure.²⁴⁹ *tert*-Butyl 3-oxo-1-phenyl-2,7,10,13-tetraoxa-4-azapentadecan-15-oate (**37**, 1.19 g, 3.00 mmol) was dissolved in dry EtOAc (30 mL) and was treated with 10% Pd/C (10% m/m). The mixture was stirred under H₂ (1 atm, balloon) for 18 h. The mixture was filtered through celite and the filtrate was

concentrated *in vacuo*. The residue was dissolved in dry DMSO (30 mL) and DIPEA (776 mg, 6.00 mmol) and 2-(2,6-dioxopiperidin-3-yl)-4-fluoroisoindoline-1,3-dione (**19**, 829 mg, 3.00 mmol) were added. The reaction mixture was stirred at 90 °C for 18 h. The mixture was allowed to cool to room temperature and was poured onto half-saturated brine (300 mL). The mixture was extracted with EtOAc (2 × 150 mL). The combined organic layers were washed with 5 % aqueous LiCl solution (150 mL) and brine (150 mL), dried over Na₂SO₄, filtered and concentrated *in vacuo*. The residue was purified by silica gel column chromatography using a gradient of petroleum ether/EtOAc (1+1) to petroleum ether/EtOAc (1+2) to yield a yellow resin (530 mg).

Yield 34%; ¹H NMR (600 MHz, DMSO-*d*₆) δ 1.41 (s, 9H, C(CH₃)₃), 2.00 – 2.05 (m, 1H, 4'-H), 2.51 – 2.62 (m, 2H, 4'-H, 5'-H), 2.84 – 2.92 (m, 1H, 5'-H), 3.47 (q, *J* = 5.6 Hz, 2H), 3.51 – 3.59 (m, 8H), 3.62 (t, *J* = 5.5 Hz, 2H), 3.96 (s, 2H, NHCH₂, OCH₂), 5.05 (dd, *J* = 12.8, 5.4 Hz, 1H, 3'-H), 6.60 (t, *J* = 5.9 Hz, 1H, NHCH₂), 7.04 (d, *J* = 7.0 Hz, 1H), 7.15 (d, *J* = 8.6 Hz, 1H, 5-H, 7-H), 7.58 (dd, *J* = 8.4, 7.2 Hz, 1H, 6-H), 11.07 (s, 1H, NH_{imide}); ¹³C NMR (151 MHz, DMSO-*d*₆) δ 22.1 (C-4'), 27.7 (C(CH₃)₃), 30.9 (C-5'), 41.7 (CH₂NH), 48.5 (C-3'), 68.1, 68.9, 69.7, 69.8 (OCH₂), 80.6 (C(CH₃)₃), 109.2 (C-3a), 110.6 (C-7), 117.4 (C-5), 132.1 (C-7a), 136.2 (C-6), 146.4 (C-4), 167.3 (C-1), 168.9 (C-3), 169.3 (C-2'), 170.0 (C-6'), 172.7 (COO), two signals (OCH₂) are missing due to overlapping signals; LC-MS (ESI) (90% H₂O to 100% MeOH in 10 min, then 100% MeOH to 20 min, DAD 220-500 nm), *t*_R = 10.27 min, 97% purity, *m/z* [M + H]⁺ calcd for C₂₅H₃₃N₃O₉ 520.23, found 520.4.

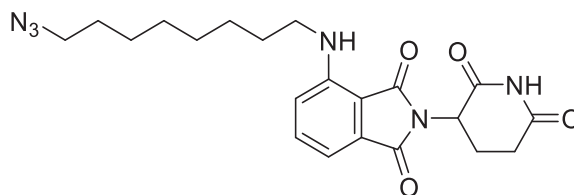
4-((6-Azidohexyl)amino)-2-(2,6-dioxopiperidin-3-yl)isoindoline-1,3-dione (**89**)^{292,348}



Compound **89** was prepared similar to a previously reported procedure.²⁹² 6-Azidohexan-1-amine (**44**, 156 mg, 1.10 mmol) was dissolved in dry DMF (8 mL). DIPEA (284 mg, 2.20 mmol) and 2-(2,6-dioxopiperidin-3-yl)-4-fluoroisoindoline-1,3-dione (**19**, 304 mg, 1.10 mmol) were added. The reaction mixture was stirred at 90 °C for 4 h. The organic layer was concentrated *in vacuo*. The residue was purified by silica gel flash column chromatography using a cyclohexane/EtOAc gradient (0% to 50% EtOAc) as eluent to yield a yellow solid (69 mg).

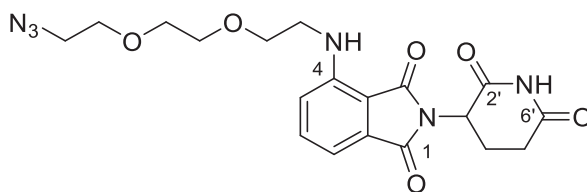
Yield 16%; mp: 98-100 °C; $^1\text{H NMR}$ (600 MHz, CDCl_3 -*d*) δ 1.42 – 1.48 (m, 4H), 1.60 – 1.65 (m, 2H), 1.66 – 1.71 (m, 2H), 2.10 – 2.16 (m, 1H), 2.69 – 2.84 (m, 2H), 2.86 – 2.92 (m, 1H), 3.25 – 3.30 (m, 4H), 4.91 (dd, J = 12.3, 5.4 Hz, 1H), 6.23 (s, 1H), 6.88 (d, J = 8.5 Hz, 1H), 7.09 (d, J = 7.1 Hz, 1H), 7.49 (dd, J = 8.5, 7.1 Hz, 1H), 8.06 (s, 1H); $^{13}\text{C NMR}$ (151 MHz, CDCl_3 -*d*) δ 22.9, 26.6, 26.7, 28.9, 29.3, 31.6, 42.6, 49.0, 51.5, 110.1, 111.6, 116.7, 132.6, 136.3, 147.1, 167.7, 168.4, 169.7, 171.1; **LC-MS** (ESI) (90% H_2O to 100% MeCN in 10 min, then 100% MeCN to 15 min, DAD 200-600 nm), t_{R} = 7.05 min, 99% purity, m/z $[\text{M} + \text{H}]^+$ calcd for $\text{C}_{19}\text{H}_{22}\text{N}_6\text{O}_4$ 399.18, found 399.3.

4-((8-Azidooctyl)amino)-2-(2,6-dioxopiperidin-3-yl)isoindoline-1,3-dione (90)²⁹³



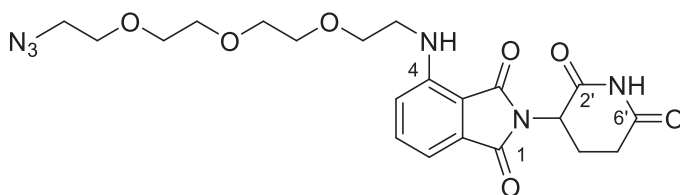
8-Azidoctan-1-amine (**47**, 341 mg, 2.00 mmol) was dissolved in dry DMF (8 mL). DIPEA (517 mg, 4.00 mmol) and 2-(2,6-dioxopiperidin-3-yl)-4-fluoroisoindoline-1,3-dione (**19**, 552 mg, 2.00 mmol) were added. The reaction mixture was stirred at 90 °C for 4 h. The organic layer was concentrated *in vacuo*. The residue was purified by silica gel flash column chromatography using a cyclohexane/EtOAc gradient (0% to 50% EtOAc) as eluent to yield a yellow resin (137 mg).

Yield 16%; $^1\text{H NMR}$ (600 MHz, CDCl_3 -*d*) δ 1.32 – 1.44 (m, 8H), 1.57 – 1.63 (m, 2H), 1.63 – 1.69 (m, 2H), 2.10 – 2.15 (m, 1H), 2.69 – 2.84 (m, 2H), 2.86 – 2.92 (m, 1H), 3.22 – 3.29 (m, 4H), 4.91 (dd, J = 12.3, 5.4 Hz, 1H), 6.23 (s, 1H), 6.88 (d, J = 8.5 Hz, 1H), 7.08 (d, J = 7.0 Hz, 1H), 7.46 – 7.53 (m, 1H), 8.09 (s, 1H); $^{13}\text{C NMR}$ (151 MHz, CDCl_3 -*d*) δ 22.9, 26.8, 27.0, 28.9, 29.2, 29.3, 29.3, 31.5, 42.8, 49.0, 51.6, 110.0, 111.5, 116.8, 132.6, 136.3, 147.1, 167.8, 168.4, 169.6, 171.1, one signal is missing due to overlapping signals; **LC-MS** (ESI) (90% H_2O to 100% MeCN in 10 min, then 100% MeCN to 15 min, DAD 200-600 nm), t_{R} = 7.98 min, 92% purity, m/z $[\text{M} + \text{H}]^+$ calcd for $\text{C}_{21}\text{H}_{26}\text{N}_6\text{O}_4$ 427.21, found 427.3.

4-((2-(2-(2-Azidoethoxy)ethoxy)ethyl)amino)-2-(2,6-dioxopiperidin-3-yl)isoindoline-1,3-dione (91)^{242,292,348}

Compound **91** was prepared similar to a previously reported procedure.²⁴² 2-(2-(2-Azidoethoxy)ethoxy)ethan-1-amine (**51**, 993 mg, 5.70 mmol) was dissolved in dry DMF (10 mL). DIPEA (1.14 g, 8.80 mmol) and 2-(2,6-dioxopiperidin-3-yl)-4-fluoroisoindoline-1,3-dione (**19**, 1.22 g, 4.40 mmol) were added. The reaction mixture was stirred at 90 °C for 4 h. The organic layer was concentrated *in vacuo*. The residue was purified by silica gel column chromatography using a gradient from petroleum ether/EtOAc (1+1) to petroleum ether/EtOAc (1+2) to yield a yellow solid (432 mg).

Yield 23%; mp: 93-95 °C; ¹H NMR (500 MHz, DMSO-*d*₆) δ 1.99 – 2.07 (m, 1H, 4'-H), 2.51 – 2.63 (m, 2H, 4'-H, 5'-H), 2.83 – 2.93 (m, 1H, 5'-H), 3.34 – 3.38 (m, 2H), 3.47 (q, *J* = 5.6 Hz, 2H), 3.57 – 3.62 (m, 6H), 3.64 (t, *J* = 5.4 Hz, 2H, CH₂N₃, OCH₂, CH₂N), 5.05 (dd, *J* = 12.7, 5.4 Hz, 1H, 3'-H), 6.60 (t, *J* = 5.9 Hz, 1H, CH₂NH), 7.04 (d, *J* = 7.0 Hz, 1H), 7.14 (d, *J* = 8.6 Hz, 1H, 5-H, 7-H), 7.58 (dd, *J* = 8.4, 7.3 Hz, 1H, 6-H), 11.06 (s, 1H, NH imide); ¹³C NMR (126 MHz, DMSO-*d*₆) δ 22.1 (C-4'), 30.9 (C-5'), 41.7 (CH₂NH), 48.5 (C-3'), 50.0 (CH₂N₃), 68.9, 69.3, 69.7, 69.8 (OCH₂), 109.3 (C-3a), 110.6 (C-7), 117.4 (C-5), 132.1 (C-7a), 136.2 (C-6), 146.4 (C-4), 167.2 (C-1), 168.9 (C-3), 170.0 (C-2'), 172.7 (C-6'); LC-MS (ESI) (90% H₂O to 100% MeOH in 10 min, then 100% MeOH to 20 min, DAD 200-800 nm), *t*_R = 8.98 min, 98% purity, *m/z* [M + H]⁺ calcd for C₁₉H₂₂N₆O₆ 431.17, found 431.2.

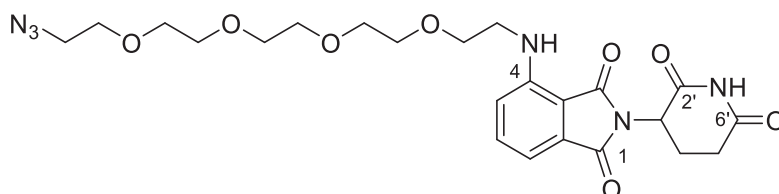
4-((2-(2-(2-(2-Azidoethoxy)ethoxy)ethoxy)ethyl)amino)-2-(2,6-dioxopiperidin-3-yl)isoindoline-1,3-dione (92)^{242,292,348}

Compound **92** was prepared similar to a previously reported procedure.²⁴² 2-(2-(2-(2-Azidoethoxy)ethoxy)ethoxy)ethan-1-amine (**55**, 993 mg, 4.55 mmol) was dissolved in dry DMF (10 mL). DIPEA (905 mg, 7.00 mmol) and 2-(2,6-dioxopiperidin-3-yl)-4-

fluoroisindoline-1,3-dione (**19**, 967 mg, 3.50 mmol) were added. The reaction mixture was stirred at 90 °C for 4 h. The organic layer was concentrated *in vacuo*. The residue was purified by silica gel flash column chromatography using a petroleum ether/EtOAc gradient (0% to 50% EtOAc) to yield a green resin (397 mg).

Yield 24%; ¹H NMR (500 MHz, DMSO-*d*₆) δ 2.00 – 2.06 (m, 1H, 4'-H), 2.51 – 2.62 (m, 2H, 4'-H, 5'-H), 2.83 – 2.93 (m, 1H, 5'-H), 3.35 – 3.38 (m, 2H), 3.45 – 3.49 (m, 2H), 3.53 – 3.59 (m, 10H), 3.63 (t, *J* = 5.4 Hz, 2H, CH₂N₃, OCH₂, CH₂N), 5.05 (dd, *J* = 12.7, 5.4 Hz, 1H, 3'-H), 6.59 (t, *J* = 5.9 Hz, 1H, CH₂NH), 7.04 (d, *J* = 7.0 Hz, 1H), 7.14 (d, *J* = 8.6 Hz, 1H, 5-H, 7-H), 7.58 (dd, *J* = 8.4, 7.2 Hz, 1H, 6-H), 11.06 (s, 1H, NH_{imide}); ¹³C NMR (126 MHz, DMSO-*d*₆) δ 22.1 (C-4'), 30.9 (C-5'), 41.7 (CH₂NH), 48.5 (C-3'), 50.0 (CH₂N₃), 68.9, 69.2, 69.7, 69.7, 69.8, 69.8 (OCH₂), 109.2 (C-3a), 110.6 (C-7), 117.4 (C-5), 132.1 (C-7a), 136.2 (C-6), 146.4 (C-4), 167.2 (C-1), 168.9 (C-3), 170.0 (C-2'), 172.7 (C-6'); LC-MS (ESI) (90% H₂O to 100% MeOH in 10 min, then 100% MeOH to 20 min, DAD 200-800 nm), *t*_R = 9.08 min, 96% purity, *m/z* [M + H]⁺ calcd for C₂₁H₂₆N₆O₇ 475.19, found 475.3.

4-((14-Azido-3,6,9,12-tetraoxatetradecyl)amino)-2-(2,6-dioxopiperidin-3-yl)isindoline-1,3-dione (93**)**^{242,292,348}

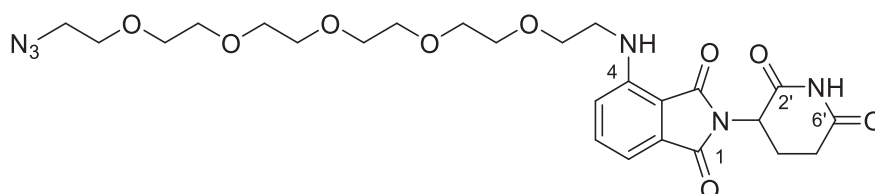


Compound **93** was prepared similar to a previously reported procedure.²⁴² 14-Azido-3,6,9,12-tetraoxatetradecan-1-amine (**59**, 1.19 g, 4.55 mmol) was dissolved in dry DMF (10 mL). DIPEA (905 mg, 7.00 mmol) and 2-(2,6-dioxopiperidin-3-yl)-4-fluoroisindoline-1,3-dione (**19**, 967 mg, 3.50 mmol) were added. The reaction mixture was stirred at 90 °C for 4 h. The organic layer was concentrated *in vacuo*. The residue was purified by silica gel flash column chromatography using a petroleum ether/EtOAc gradient (0% to 50% EtOAc) as eluent to yield a green resin (288 mg).

Yield 16%; ¹H NMR (500 MHz, DMSO-*d*₆) δ 1.99 – 2.07 (m, 1H, 4'-H), 2.52 – 2.63 (m, 2H, 4'-H, 5'-H), 2.83 – 2.93 (m, 1H, 5'-H), 3.36 – 3.39 (m, 2H), 3.45 – 3.49 (m, 2H), 3.50 – 3.55 (m, 10H), 3.56 – 3.60 (m, 4H), 3.63 (t, *J* = 5.4 Hz, 2H, CH₂N₃, OCH₂, CH₂N), 5.05 (dd, *J* = 12.7, 5.4 Hz, 1H, 3'-H), 6.59 (t, *J* = 5.9 Hz, 1H, CH₂NH), 7.04 (d, *J* = 7.0 Hz, 1H), 7.14 (d, *J* = 8.6 Hz, 1H, 5-H, 7-H), 7.58 (dd, *J* = 8.6, 7.1 Hz, 1H, 6-H), 11.06 (s, 1H, NH_{imide}); ¹³C NMR

(126 MHz, DMSO- d_6) δ 22.1 (C-4'), 30.9 (C-5'), 41.7 (CH₂NH), 48.5 (C-3'), 50.0 (CH₂N₃), 68.9, 69.2, 69.6, 69.7, 69.8, 69.8, 69.8 (OCH₂), 109.2 (C-3a), 110.6 (C-7), 117.4 (C-5), 132.1 (C-7a), 136.2 (C-6), 146.4 (C-4), 167.2 (C-1), 168.9 (C-3), 170.0 (C-2'), 172.7 (C-6'), one signal (OCH₂) is missing due to overlapping signals; **LC-MS** (ESI) (90% H₂O to 100% MeCN in 10 min, then 100% MeCN to 15 min, DAD 200-600 nm), t_R = 5.59 min, 97% purity, m/z [M + H]⁺ calcd for C₂₃H₃₀N₆O₈ 519.22, found 519.4.

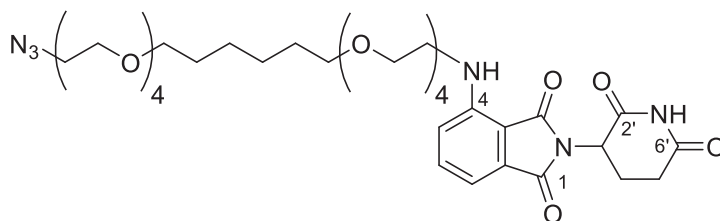
4-((17-Azido-3,6,9,12,15-pentaoxaheptadecyl)amino)-2-(2,6-dioxopiperidin-3-yl)isoindoline-1,3-dione (94)³⁴⁸



17-Azido-3,6,9,12,15-pentaoxaheptadecan-1-amine (**63**, 1.39 g, 4.55 mmol) was dissolved in dry DMF (10 mL). DIPEA (905 mg, 7.00 mmol) and 2-(2,6-dioxopiperidin-3-yl)-4-fluoroisoindoline-1,3-dione (**19**, 967 mg, 3.50 mmol) were added. The reaction mixture was stirred at 90 °C for 4 h. The organic layer was concentrated *in vacuo*. The residue was purified by silica gel flash column chromatography using a cyclohexane/EtOAc gradient (0% to 100% EtOAc) as eluent to yield a green resin (281 mg).

Yield 14%; ¹H NMR (500 MHz, DMSO- d_6) δ 1.97 – 2.07 (m, 1H, 4'-H), 2.51 – 2.65 (m, 2H, 4'-H, 5'-H), 2.83 – 2.96 (m, 1H, 5'-H), 3.36 – 3.40 (m, 2H), 3.45 – 3.60 (m, 20H), 3.63 (t, J = 5.4 Hz, 2H, CH₂N₃, OCH₂, CH₂N), 5.05 (dd, J = 12.7, 5.5 Hz, 1H, 3'-H), 6.60 (t, J = 5.9 Hz, 1H, CH₂NH), 7.04 (d, J = 7.0 Hz, 1H), 7.14 (d, J = 8.6 Hz, 1H, 5-H, 7-H), 7.58 (dd, J = 8.4, 7.2 Hz, 1H, 6-H), 11.06 (s, 1H, NH imide); ¹³C NMR (126 MHz, DMSO- d_6) δ 22.1 (C-4'), 30.9 (C-5'), 41.7 (CH₂NH), 48.5 (C-3'), 50.0 (CH₂N₃), 68.9, 69.2, 69.7, 69.7, 69.7, 69.8, 69.8 (OCH₂), 109.2 (C-3a), 110.6 (C-7), 117.4 (C-5), 132.1 (C-7a), 136.2 (C-6), 146.4 (C-4), 167.2 (C-1), 168.9 (C-3), 170.0 (C-2'), 172.7 (C-6'), three signals (OCH₂) are missing due to overlapping signals; **LC-MS** (ESI) (90% H₂O to 100% MeCN in 10 min, then 100% MeCN to 15 min, DAD 200-600 nm), t_R = 5.60 min, 90% purity, m/z [M + H]⁺ calcd for C₂₅H₃₄N₆O₉ 563.25, found 563.4.

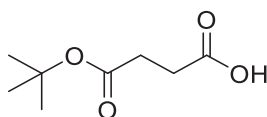
4-((30-Azido-3,6,9,12,19,22,25,28-octaoxatriacontyl)amino)-2-(2,6-dioxopiperidin-3-yl)isoindoline-1,3-dione (95)



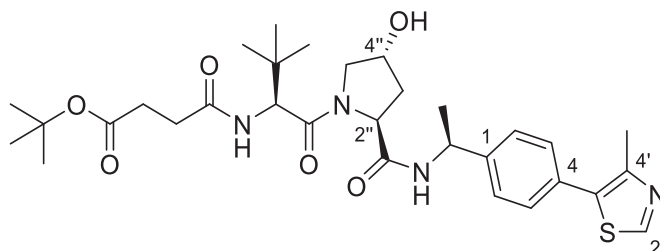
30-Azido-3,6,9,12,19,22,25,28-octaoxatriacontan-1-amine (**67**, 544 mg, 1.10 mmol) was dissolved in dry DMF (8 mL). DIPEA (284 mg, 2.20 mmol) and 2-(2,6-dioxopiperidin-3-yl)-4-fluoroisoindoline-1,3-dione (**19**, 304 mg, 1.10 mmol) were added. The reaction mixture was stirred at 90 °C for 4 h. The organic layer was concentrated *in vacuo*. The residue was purified by silica gel flash column chromatography using a CH₂Cl₂/MeOH gradient (0% to 5% MeOH) to yield a yellow oil (171 mg).

Yield 21%; ¹H NMR (600 MHz, DMSO-*d*₆) δ 1.22 – 1.29 (m, 4H, CH₂), 1.42 – 1.48 (m, 4H, CH₂), 1.98 – 2.04 (m, 1H, 4'-H), 2.50 – 2.61 (m, 2H, 4'-H, 5'-H), 2.84 – 2.91 (m, 1H, 5'-H), 3.31 – 3.35 (m, 4H), 3.36 – 3.39 (m, 2H), 3.41 – 3.57 (m, 26H), 3.57 – 3.60 (m, 2H), 3.61 (t, *J* = 5.5 Hz, 2H, CH₂N₃, OCH₂, CH₂N), 5.04 (dd, *J* = 12.8, 5.4 Hz, 1H, 3'-H), 6.59 (t, *J* = 5.9 Hz, 1H, CH₂NH), 7.03 (d, *J* = 7.0 Hz, 1H), 7.14 (d, *J* = 8.6 Hz, 1H, 5-H, 7-H), 7.57 (dd, *J* = 8.4, 7.2 Hz, 1H, 6-H), 11.06 (s, 1H, NH imide); ¹³C NMR (151 MHz, DMSO-*d*₆) δ 22.1 (C-4'), 25.5, 29.2 (CH₂), 30.9 (C-5'), 41.7 (CH₂NH), 48.5 (C-3'), 50.0 (CH₂N₃), 68.9, 69.2, 69.4, 69.4, 69.7, 69.8, 69.8, 69.8, 69.8, 70.2 (OCH₂), 109.2 (C-3a), 110.6 (C-7), 117.4 (C-5), 132.1 (C-7a), 136.2 (C-6), 146.4 (C-4), 167.2 (C-1), 168.9 (C-3), 170.0 (C-2'), 172.7 (C-6'); LC-MS (ESI) (90% H₂O to 100% MeCN in 10 min, then 100% MeCN to 15 min, DAD 200-600 nm), *t*_R = 6.79 min, 93% purity, *m/z* [M + NH₄]⁺ calcd for C₃₅H₅₄N₆O₁₂ 768.41, found 768.6.

4-(*tert*-Butoxy)-4-oxobutanoic acid (97)



Compound **97** was purchased from TCI (Eschborn, Germany).

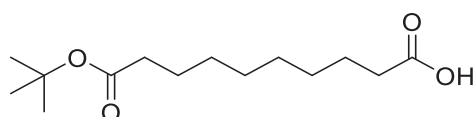
tert*-Butyl*4-(((*S*)-1-((2*S*,4*R*)-4-hydroxy-2-(((*S*)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)carbamoyl)pyrrolidin-1-yl)-3,3-dimethyl-1-oxobutan-2-yl)amino)-4-oxobutanoate (**98**)**

The VHL ligand **26** (545 mg, 1.00 mmol) was dissolved in dry CH₂Cl₂ (15 mL) and treated with TFA (5 mL). The mixture was stirred for 2 h. The mixture was concentrated *in vacuo*. 4-(*tert*-Butoxy)-4-oxobutanoic acid (**97**, 174 mg, 1.00 mmol) was dissolved in dry DMF (5 mL). HATU (418 mg, 1.10 mmol) and DIPEA (452 mg, 3.50 mmol) were added under argon. The deprotected VHL ligand **96** was dissolved in dry DMF (5 mL) and was added to the mixture, containing the activated acid compound. The reaction mixture was stirred for 18 h at room temperature under argon. The reaction was quenched by the addition of water (25 mL) and was extracted with EtOAc (3 × 50 mL). The combined organic layers were washed with brine (75 mL), dried over Na₂SO₄, filtered and concentrated *in vacuo*. The residue was purified by silica gel column chromatography using CH₂Cl₂/MeOH (19+1) as eluent to yield a colorless resin (486 mg).

Yield 81%; ¹H NMR (600 MHz, DMSO-*d*₆) δ 0.93 (s, 9H, CHC(CH₃)₃), 1.36 – 1.39 (m, 12H, OC(CH₃)₃, CHCH₃), 1.77 – 1.83 (m, 1H), 1.97 – 2.03 (m, 1H, 3''-H), 2.26 – 2.44 (m, 4H, CH₂), 2.45 (s, 3H, CH₃ thiazole), 3.54 – 3.58 (m, 1H), 3.60 – 3.64 (m, 1H, 5''-H), 4.25 – 4.31 (m, 1H), 4.42 (t, *J* = 8.0 Hz, 1H), 4.52 (d, *J* = 9.3 Hz, 1H, 2''-H, 4''-H, NHCH), 4.88 – 4.95 (m, 1H, CHCH₃), 5.07 (d, *J* = 3.6 Hz, 1H, OH), 7.36 – 7.40 (m, 2H), 7.41 – 7.45 (m, 2H, 2-H, 3-H, 5-H, 6-H), 7.86 (d, *J* = 9.3 Hz, 1H), 8.35 (d, *J* = 7.8 Hz, 1H, CONH), 8.98 (s, 1H, 2'-H); ¹³C NMR (151 MHz, DMSO-*d*₆) δ 15.9 (CH₃ thiazole), 22.4 (CHCH₃), 26.4 (CHC(CH₃)₃), 27.7 OC(CH₃)₃, 29.8, 30.4 (CH₂), 35.3 (CHC(CH₃)₃), 37.7 (C-3''), 47.7 (CHCH₃), 56.2, 56.4, 58.5 (C-2'', C-5'', NHCH), 68.7 (C-4''), 79.4 (OC(CH₃)₃), 126.3, 128.8 (C-2, C-3, C-5, C-6), 129.7, 131.1, 144.6 (C-1, C-4, C-5'), 147.7 (C-4'), 151.4 (C-2'), 169.4, 170.6, 170.6, 171.5 (CO); LC-MS (ESI) (90% H₂O to 100% MeOH in 10 min, then 100% MeOH to 20 min, DAD 220-400 nm), *t*_R = 11.35 min, 99% purity, *m/z* [M + H]⁺ calcd for C₃₁H₄₄N₄O₆S 601.31, found 601.5.

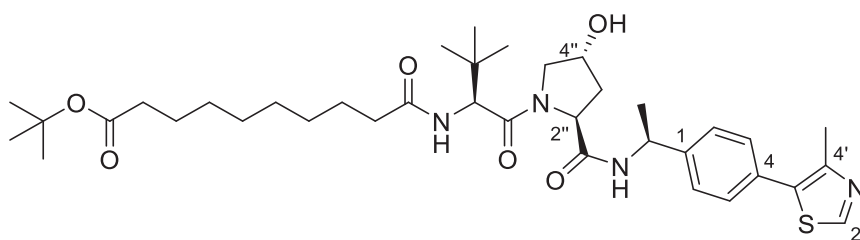
(CH₂), 35.2 (CHC(CH₃)₃), 37.7 (C-3''), 47.7 (CHCH₃), 56.2, 56.4, 58.5 (C-2'', C-5'', NHCH), 68.7 (C-4''), 79.3 (OC(CH₃)₃), 126.4, 128.8 (C-2, C-3, C-5, C-6), 129.7, 131.1, 144.6 (C-1, C-4, C-5'), 147.8 (C-4'), 151.4 (C-2'), 169.6, 170.6, 172.1, 172.3 (CO); **LC-MS** (ESI) (90% H₂O to 100% MeOH in 10 min, then 100% MeOH to 20 min, DAD 220-400 nm), *t_R* = 11.96 min, 95% purity, *m/z* [M + H]⁺ calcd for C₃₅H₅₂N₄O₆S 657.37, found 657.4.

10-(*tert*-Butoxy)-10-oxodecanoic acid (**101**)



Compound **101** was purchased from chempur (Karlsruhe, Germany).

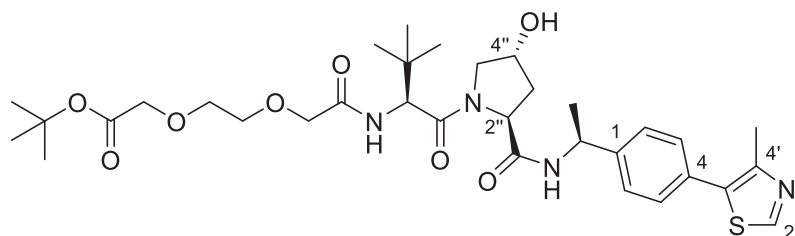
tert-Butyl 10-((((*S*)-1-((2*S*,4*R*)-4-hydroxy-2-((((*S*)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)carbamoyl)pyrrolidin-1-yl)-3,3-dimethyl-1-oxobutan-2-yl)amino)-10-oxodecanoate (**102**)



The VHL ligand **26** (545 mg, 1.00 mmol) was dissolved in dry CH₂Cl₂ (15 mL) and treated with TFA (5 mL). The mixture was stirred for 2 h. The mixture was concentrated *in vacuo*. 10-(*tert*-Butoxy)-10-oxodecanoic acid (**101**, 258 mg, 1.00 mmol) was dissolved in dry DMF (5 mL). HATU (418 mg, 1.10 mmol) and DIPEA (452 mg, 3.50 mmol) were added under argon. The deprotected VHL ligand **96** was dissolved in dry DMF (5 mL) and was added to the mixture, containing the activated acid compound. The reaction mixture was stirred for 18 h at room temperature under argon. The reaction was quenched by the addition of water (25 mL) and was extracted with EtOAc (3 × 50 mL). The combined organic layers were washed with brine (75 mL), dried over Na₂SO₄, filtered and concentrated *in vacuo*. The residue was purified by silica gel column chromatography using CH₂Cl₂/MeOH (19+1) as eluent to yield a white solid (471 mg).

Yield 69%; mp: 98-100 °C; ^1H NMR (600 MHz, DMSO- d_6) δ 0.93 (s, 9H, $\text{CHC}(\text{CH}_3)_3$), 1.21 – 1.26 (m, 8H, CH_2), 1.36 – 1.39 (m, 12H, $\text{OC}(\text{CH}_3)_3$, CHCH_3), 1.41 – 1.53 (m, 4H, CH_2), 1.76 – 1.83 (m, 1H), 1.97 – 2.03 (m, 1H, 3''-H), 2.07 – 2.12 (m, 1H), 2.13 – 2.18 (m, 2H), 2.20 – 2.28 (m, 1H, CH_2), 2.45 (s, 3H, CH_3 thiazole), 3.56 – 3.64 (m, 2H, 5''-H), 4.25 – 4.30 (m, 1H), 4.42 (t, J = 8.0 Hz, 1H), 4.52 (d, J = 9.3 Hz, 1H, 2''-H, 4''-H, NHCH), 4.89 – 4.95 (m, 1H, CHCH_3), 5.07 (d, J = 3.6 Hz, 1H, OH), 7.32 – 7.40 (m, 2H), 7.41 – 7.45 (m, 2H, 2-H, 3-H, 5-H, 6-H), 7.75 (d, J = 9.3 Hz, 1H), 8.34 (d, J = 7.8 Hz, 1H, CONH), 8.98 (s, 1H, 2'-H); ^{13}C NMR (151 MHz, DMSO- d_6) δ 15.9 (CH_3 thiazole), 22.4 (CHCH_3), 24.6, 25.4 (CH_2), 26.4 ($\text{CHC}(\text{CH}_3)_3$), 27.8 ($\text{OC}(\text{CH}_3)_3$), 28.3, 28.6, 34.8, 34.8 (CH_2), 35.2 ($\text{CHC}(\text{CH}_3)_3$), 37.7 (3''-H), 47.7 (CHCH_3), 56.2, 56.3, 58.5 (C-2'', C-5'', NHCH), 68.7 (C-4''), 79.3 ($\text{OC}(\text{CH}_3)_3$), 126.4, 128.8 (C-2, C-3, C-5, C-6), 129.7, 131.1, 144.6 (C-1, C-4, C-5'), 147.7 (C-4'), 151.4 (C-2'), 169.6, 170.6, 172.0, 172.3 (CO); LC-MS (ESI) (90% H_2O to 100% MeOH in 10 min, then 100% MeOH to 20 min, DAD 220-400 nm), t_R = 12.40 min, 98% purity, m/z $[\text{M} + \text{H}]^+$ calcd for $\text{C}_{37}\text{H}_{56}\text{N}_4\text{O}_6\text{S}$ 685.40, found 685.7.

***tert*-Butyl 2-(2-(2-(((*S*)-1-((2*S*,4*R*)-4-hydroxy-2-(((*S*)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)carbamoyl)pyrrolidin-1-yl)-3,3-dimethyl-1-oxobutan-2-yl)amino)-2-oxoethoxy)ethoxy)acetate (103)**

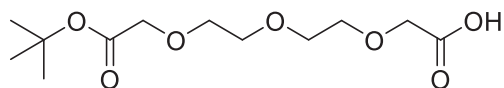


The VHL ligand **26** (545 mg, 1.00 mmol) was dissolved in dry CH_2Cl_2 (15 mL) and treated with TFA (5 mL). The mixture was stirred for 2 h. The mixture was concentrated *in vacuo*. 2-(2-(2-(*tert*-Butoxy)-2-oxoethoxy)ethoxy)acetic acid (**41**, 234 mg, 1.00 mmol) was dissolved in dry DMF (5 mL). HATU (418 mg, 1.10 mmol) and DIPEA (452 mg, 3.50 mmol) were added under argon. The deprotected VHL ligand **96** was dissolved in dry DMF (5 mL) and was added to the mixture, containing the activated acid compound. The reaction mixture was stirred for 18 h at room temperature under argon. The reaction was quenched by the addition of water (25 mL) and was extracted with EtOAc (3×50 mL). The combined organic layers were washed with brine (75 mL), dried over Na_2SO_4 , filtered and concentrated *in vacuo*. The residue was

purified by silica gel column chromatography using CH₂Cl₂/MeOH (19+1) as eluent to yield a white solid (395 mg).

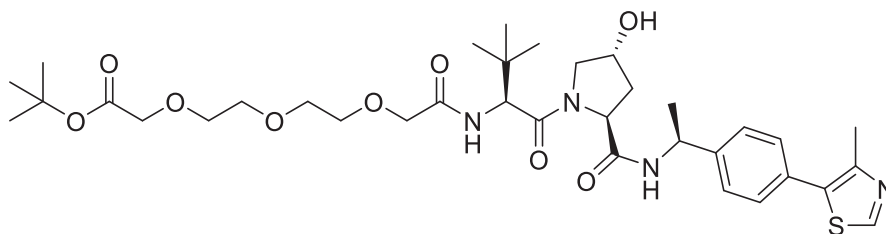
Yield 60%; mp: 49-53 °C; ¹H NMR (600 MHz, DMSO-*d*₆) δ 0.94 (s, 9H, CHC(CH₃)₃), 1.38 (d, *J* = 7.0 Hz, 3H, CHCH₃), 1.42 (s, 9H, OC(CH₃)₃), 1.75 – 1.82 (m, 1H), 2.01 – 2.07 (m, 1H, 3''-H), 2.45 (s, 3H, CH₃ thiazole), 3.54 – 3.67 (m, 6H, 5''-H, CH₂), 3.95 – 4.09 (m, 4H, CH₂), 4.26 – 4.30 (m, 1H), 4.42 – 4.47 (m, 1H), 4.55 (d, *J* = 9.5 Hz, 1H, 2''-H, 4''-H, NHCH), 4.88 – 4.94 (m, 1H, CHCH₃), 5.11 (d, *J* = 3.6 Hz, 1H, OH), 7.34 – 7.39 (m, 2H), 7.39 – 7.46 (m, 3H, 2-H, 3-H, 5-H, 6-H, CONH), 8.41 (d, *J* = 7.7 Hz, 1H, CONH), 8.98 (s, 1H, 2'-H); ¹³C NMR (151 MHz, DMSO-*d*₆) δ 15.9 (CH₃ thiazole), 22.4 (CHCH₃), 26.2 (CHC(CH₃)₃), 27.7 (OC(CH₃)₃), 35.7 (CHC(CH₃)₃), 37.7 (C-3''), 47.7 (CHCH₃), 55.7, 56.5, 58.5 (C-2'', C-5'', NHCH), 68.1, 68.7, 69.6, 69.7, 70.4 (CH₂, C-4''), 80.7 (OC(CH₃)₃), 126.3, 128.8 (C-2, C-3, C-5, C-6), 129.7, 131.1, 144.7 (C-1, C-4, C-5'), 147.7 (C-4'), 151.4 (C-2'), 168.5, 169.0, 169.3, 170.4 (CO); LC-MS (ESI) (90% H₂O to 100% MeOH in 10 min, then 100% MeOH to 20 min, DAD 220-400 nm), *t*_R = 11.46 min, 100% purity, *m/z* [M + H]⁺ calcd for C₃₃H₄₈N₄O₈S 661.33, found 661.6.

13,13-Dimethyl-11-oxo-3,6,9,12-tetraoxatetradecanoic acid (104)

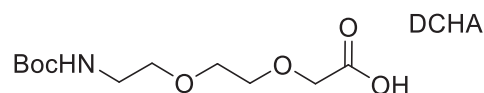


Compound **104** was prepared as described before.³²³

tert-Butyl (S)-13-((2*S*,4*R*)-4-hydroxy-2-(((S)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)carbamoyl)pyrrolidine-1-carbonyl)-14,14-dimethyl-11-oxo-3,6,9-trioxa-12-azapentadecanoate (105)

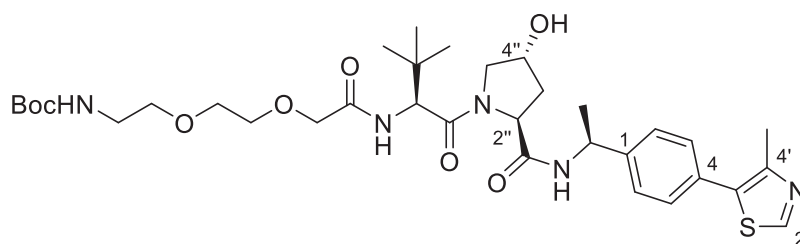


Compound **105** was prepared as described before.³²³

2,2-Dimethyl-4-oxo-3,8,11-trioxa-5-azatridecan-13-oic acid × DCHA (106)

Compound **106** was purchased from chempur (Karlsruhe, Germany).

tert-Butyl (2-(2-(2-(((S)-1-((2S,4R)-4-hydroxy-2-(((S)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)carbamoyl)pyrrolidin-1-yl)-3,3-dimethyl-1-oxobutan-2-yl)amino)-2-oxoethoxy)ethoxy)ethyl)carbamate (107)

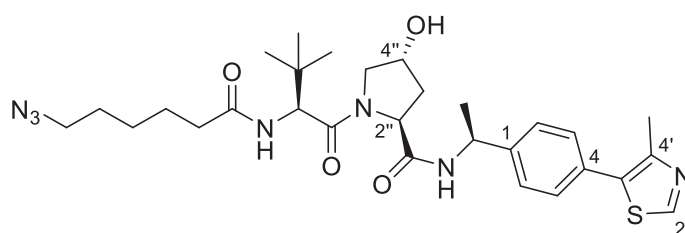


The VHL ligand **26** (545 mg, 1.00 mmol) was dissolved in dry CH_2Cl_2 (15 mL) and treated with TFA (5 mL). The mixture was stirred for 2 h. The mixture was concentrated *in vacuo*. 2,2-Dimethyl-4-oxo-3,8,11-trioxa-5-azatridecan-13-oic acid × DCHA (**106**, 445 mg, 1.00 mmol) was dissolved in dry DMF (5 mL). HATU (418 mg, 1.10 mmol) and DIPEA (452 mg, 3.50 mmol) were added under argon. The deprotected VHL ligand **96** was dissolved in dry DMF (5 mL) and was added to the mixture, containing the activated acid compound. The reaction mixture was stirred for 18 h at room temperature under argon. The reaction was quenched by the addition of water (25 mL). The mixture was extracted with EtOAc (3×50 mL). The combined organic layers were washed with brine (75 mL), dried over Na_2SO_4 , filtered and concentrated *in vacuo*. The residue was purified by silica gel column chromatography using $\text{CH}_2\text{Cl}_2/\text{MeOH}$ (19+1) as eluent to yield a white solid (475 mg).

Yield 69%; mp: 46-50 °C; ^1H NMR (600 MHz, $\text{DMSO}-d_6$) δ 0.94 (s, 9H, $\text{CHC}(\text{CH}_3)_3$), 1.32 – 1.41 (m, 12H, $\text{OC}(\text{CH}_3)_3$, CHCH_3), 1.73 – 1.83 (m, 1H), 2.00 – 2.08 (m, 1H, 3''-H), 2.46 (s, 3H, CH_3 thiazole), 3.05 – 3.13 (m, 2H), 3.39 – 3.44 (m, 2H), 3.50 – 3.63 (m, 6H, CH_2 , 5''-H), 3.96 (s, 2H, OCH_2CON), 4.27 – 4.31 (m, 1H), 4.45 (t, $J = 8.1$ Hz, 1H), 4.55 (d, $J = 9.5$ Hz, 1H, 2''-H, 4''-H, NHCH), 4.88 – 4.94 (m, 1H, CHCH_3), 6.68 – 6.85 (m, 1H, NHCOO), 7.33 – 7.41 (m, 3H), 7.41 – 7.46 (m, 2H, 2-H, 3-H, 5-H, 6-H, CONH), 8.40 (d, $J = 7.7$ Hz, 1H, CONH), 9.00 (s, 1H, 2'-H), one signal (1H, OH) is missing; ^{13}C NMR (151 MHz, $\text{DMSO}-d_6$) δ 15.9 (CH_3 thiazole), 22.4 (CHCH_3), 26.2 ($\text{CHC}(\text{CH}_3)_3$), 28.2 ($\text{OC}(\text{CH}_3)_3$), 35.7 ($\text{CHC}(\text{CH}_3)_3$), 37.7 (C-

3''), 47.7 ($\underline{\text{CHCH}_3}$), 55.7, 56.5, 58.5 (C-2'', C-5'', NHCH), 68.7 (C-4''), 69.3, 69.3, 69.6, 70.4 (OCH_2), 77.5 ($\text{OC}(\underline{\text{CH}_3})_3$), 126.3, 128.8 (C-2, C-3, C-5, C-6), 129.6, 131.1, 144.7 (C-1, C-4, C-5'), 147.6 (C-4'), 151.5 (C-2'), 155.5 (NHCOO), 168.5, 169.0, 170.4 (CO), one signal (NHCH_2) is obscured by solvent signal; **LC-MS** (ESI) (90% H_2O to 100% MeOH in 10 min, then 100% MeOH to 20 min, DAD 220-400 nm), t_R = 11.48 min, 97% purity, m/z $[\text{M} + \text{H}]^+$ calcd for $\text{C}_{34}\text{H}_{51}\text{N}_5\text{O}_8\text{S}$ 690.35, found 690.6.

(2S,4R)-1-((S)-2-(6-Azidohexanamido)-3,3-dimethylbutanoyl)-4-hydroxy-N-((S)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)pyrrolidine-2-carboxamide (108)

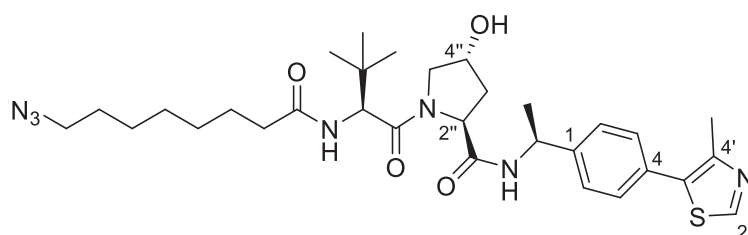


The Boc-protected VHL ligand **26** (872 mg, 1.60 mmol) was dissolved in dry CH_2Cl_2 (15 mL) and was treated with TFA (5 mL). The mixture was stirred for 2 h at room temperature. The mixture was concentrated *in vacuo*. 6-Azidohexanoic acid (**69**, 251 mg, 1.60 mmol) was dissolved in dry DMF (5 mL). HATU (669 mg, 1.76 mmol) and DIPEA (724 mg, 5.60 mmol) were added under argon. The mixture was allowed to stir for 30 min. The deprotected VHL ligand **96** was dissolved in dry DMF (5 mL) and was added to the mixture, containing the activated acid compound. The reaction mixture was allowed to stir at room temperature under argon for 4 h. The reaction mixture was concentrated *in vacuo*. The crude product was purified by silica gel flash column chromatography using a $\text{CH}_2\text{Cl}_2/\text{MeOH}$ gradient (0% to 5% MeOH) as eluent to yield a colorless resin (491 mg).

Yield 53%; $^1\text{H NMR}$ (500 MHz, $\text{DMSO}-d_6$) δ 0.94 (s, 9H, $\text{C}(\text{CH}_3)_3$), 1.28 – 1.33 (m, 2H, CH_2), 1.38 (d, J = 6.9 Hz, 3H, CHCH_3), 1.45 – 1.56 (m, 4H, CH_2), 1.76 – 1.85 (m, 1H), 1.96 – 2.05 (m, 1H, 3''-H), 2.10 – 2.17 (m, 1H), 2.22 – 2.29 (m, 1H, CH_2CON), 2.45 (s, 3H, CH_3 thiazole), 3.58 – 3.64 (m, 2H, 5''-H), 4.26 – 4.32 (m, 1H), 4.43 (t, J = 8.0 Hz, 1H), 4.52 (d, J = 9.3 Hz, 1H, 2''-H, 4''-H, NHCH), 4.88 – 4.97 (m, 1H, CHCH_3), 5.08 (d, J = 3.6 Hz, 1H, OH), 7.36 – 7.40 (m, 2H), 7.41 – 7.45 (m, 2H, 2-H, 3-H, 5-H, 6-H), 7.78 (d, J = 9.2 Hz, 1H, CONH), 8.34 (d, J = 7.8 Hz, 1H, CONH), 8.97 (s, 1H, 2'-H), one signal (2H, CH_2N_3) is obscured by solvent signal; $^{13}\text{C NMR}$ (126 MHz, $\text{DMSO}-d_6$) δ 15.9 (CH_3 thiazole), 22.3 (CHCH_3), 24.9, 25.7 (CH_2), 26.4 ($\text{C}(\text{CH}_3)_3$), 27.9(CH_2), 34.7, 35.1 (CH_2 , $\text{C}(\text{CH}_3)_3$), 37.7 (C-3''), 47.7 (CHCH_3), 50.5

(CH₂N₃), 56.2, 56.3, 58.5 (C-2'', C-5'', NHCH), 68.7 (C-4''), 126.3, 128.8 (C-2, C-3, C-5, C-6), 129.7, 131.1, 144.6 (C-1, C-4, C-5'), 147.7 (C-4'), 151.4 (C-2'), 169.6, 170.6, 171.9 (CO); **LC-MS** (ESI) (90% H₂O to 100% MeCN in 10 min, then 100% MeCN to 15 min, DAD 200-600 nm), *t_R* = 6.16 min, 92% purity, *m/z* [M + H]⁺ calcd for C₂₉H₄₁N₇O₄S 584.30, found 584.4.

(2*S*,4*R*)-1-((*S*)-2-(8-Azidooctanamido)-3,3-dimethylbutanoyl)-4-hydroxy-*N*-((*S*)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)pyrrolidine-2-carboxamide (109)

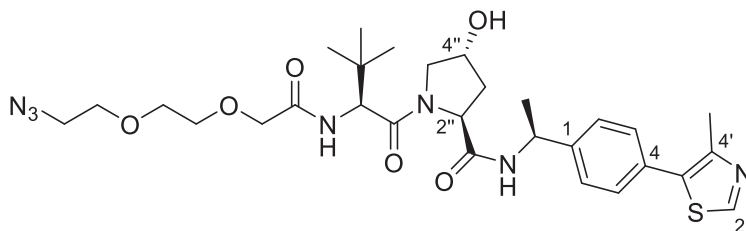


The Boc-protected VHL ligand **26** (872 mg, 1.60 mmol) was dissolved in dry CH₂Cl₂ (15 mL) and was treated with TFA (5 mL). The mixture was stirred for 2 h at room temperature. The mixture was concentrated *in vacuo*. 8-Azido-octanoic acid (**71**, 296 mg, 1.60 mmol) was dissolved in dry DMF (5 mL). HATU (669 mg, 1.76 mmol) and DIPEA (724 mg, 5.60 mmol) were added under argon. The mixture was allowed to stir for 30 min. The deprotected VHL ligand **96** was dissolved in dry DMF (5 mL) and was added to the mixture, containing the activated acid compound. The reaction mixture was allowed to stir at room temperature under argon for 4 h. The reaction mixture was concentrated *in vacuo*. The crude product was purified by silica gel flash column chromatography using a petroleum ether/EtOAc gradient (0% to 100% EtOAc) as eluent to yield a colorless resin (436 mg).

Yield 45%; ¹H NMR (600 MHz, DMSO-*d*₆) δ 0.94 (s, 9H, C(CH₃)₃), 1.26 – 1.32 (m, 6H, CH₂), 1.38 (d, *J* = 7.0 Hz, 3H, CHCH₃), 1.41 – 1.55 (m, 4H, CH₂), 1.77 – 1.83 (m, 1H), 1.98 – 2.04 (m, 1H, 3''-H), 2.08 – 2.14 (m, 1H), 2.22 – 2.28 (m, 1H, CH₂CON), 2.45 (s, 3H, CH₃ thiazole), 3.59 – 3.63 (m, 2H, 5''-H), 4.25 – 4.31 (m, 1H), 4.42 (t, *J* = 8.0 Hz, 1H), 4.52 (d, *J* = 9.3 Hz, 1H, 2''-H, 4''-H, NHCH), 4.85 – 4.96 (m, 1H, CHCH₃), 5.08 (d, *J* = 3.6 Hz, 1H, OH), 7.36 – 7.40 (m, 2H), 7.40 – 7.46 (m, 2H, 2-H, 3-H, 5-H, 6-H), 7.76 (d, *J* = 9.3 Hz, 1H, CONH), 8.35 (d, *J* = 7.8 Hz, 1H, CONH), 8.98 (s, 1H, 2'-H), one signal (2H, CH₂N₃) is obscured by solvent signal; ¹³C NMR (151 MHz, DMSO-*d*₆) δ 16.0 (CH₃ thiazole), 22.4 (CHCH₃), 25.3, 26.0 (CH₂), 26.4 (C(CH₃)₃), 28.2, 28.2, 28.5 (CH₂), 34.8, 35.2 (CH₂, C(CH₃)₃), 37.7 (C-3''), 47.7 (CHCH₃), 50.6 (CH₂N₃), 56.2, 56.3, 58.5 (C-2'', C-5'', NHCH), 68.7 (C-4''), 126.4, 128.8 (C-2, C-3, C-5, C-6), 129.7, 131.1, 144.6 (C-1, C-4, C-5'), 147.7 (C-4'), 151.4 (C-2'), 169.6, 170.6, 172.0

(CO); LC-MS (ESI) (90% H₂O to 100% MeCN in 10 min, then 100% MeCN to 15 min, DAD 200-600 nm), t_R = 6.88 min, 95% purity, m/z [M + H]⁺ calcd for C₃₁H₄₅N₇O₄S 612.33, found 612.5.

(2*S*,4*R*)-1-((*S*)-2-(2-(2-(2-Azidoethoxy)ethoxy)acetamido)-3,3-dimethylbutanoyl)-4-hydroxy-*N*-((*S*)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)pyrrolidine-2-carboxamide (110)

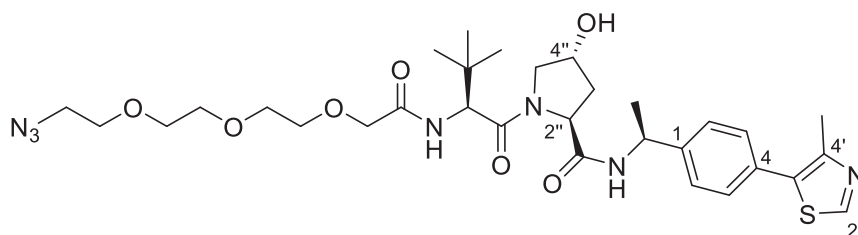


The Boc-protected VHL ligand **26** (872 mg, 1.60 mmol) was dissolved in dry CH₂Cl₂ (15 mL) and was treated with TFA (5 mL). The mixture was stirred for 2 h at room temperature. The mixture was concentrated *in vacuo*. *tert*-Butyl 2-(2-(2-azidoethoxy)ethoxy)acetate (**74**, 392 mg, 1.60 mmol) was dissolved in dry CH₂Cl₂ (15 mL) and was treated with TFA (5 mL). The mixture was stirred for 2 h at room temperature. The mixture was concentrated *in vacuo*. The deprotected linker was dissolved in dry DMF (5 mL). HATU (669 mg, 1.76 mmol) and DIPEA (724 mg, 5.60 mmol) were added under argon. The mixture was allowed to stir for 30 min. The deprotected VHL ligand **96** was dissolved in dry DMF (5 mL) and was added to the mixture, containing the activated acid compound. The reaction mixture was allowed to stir at room temperature under argon for 4 h. The reaction was quenched by adding H₂O (25 mL) and was extracted with EtOAc (3 × 50 mL). The combined organic layers were washed with brine (75 mL), dried over Na₂SO₄, filtered and concentrated *in vacuo*. The crude product was purified by silica gel column chromatography using CH₂Cl₂/MeOH (19+1) as eluent to yield a colorless resin (403 mg).

Yield 41%; ¹H NMR (500 MHz, DMSO-*d*₆) δ 0.95 (s, 9H, C(CH₃)₃), 1.38 (d, J = 7.0 Hz, 3H, CHCH₃), 1.75 – 1.82 (m, 1H), 2.01 – 2.08 (m, 1H, 3''-H), 2.46 (s, 3H, CH₃ thiazole), 3.41 – 3.45 (m, 2H), 3.55 – 3.66 (m, 8H, 5''-H, CH₂), 3.91 – 4.01 (m, 2H, CH₂CON), 4.26 – 4.31 (m, 1H), 4.45 (t, J = 8.2 Hz, 1H), 4.55 (d, J = 9.6 Hz, 1H, 2''-H, 4''-H, NHCH), 4.88 – 4.95 (m, 1H, CHCH₃), 5.10 (d, J = 3.6 Hz, 1H, OH), 7.33 – 7.39 (m, 3H), 7.41 – 7.46 (m, 2H, 2-H, 3-H, 5-H, 6-H, CONH), 8.39 (d, J = 7.7 Hz, 1H, CONH), 8.97 (s, 1H, 2'-H); ¹³C NMR (126 MHz, DMSO-*d*₆) δ 15.9 (CH₃ thiazole), 22.4 (CHCH₃), 26.2 (C(CH₃)₃), 35.7 (C(CH₃)₃), 37.7 (C-3''), 47.7 (CHCH₃), 49.9 (CH₂N₃), 55.7, 56.5, 58.5 (C-2'', C-5'', NHCH), 68.7 (C-4''), 69.3, 69.4,

69.7, 70.4 (OCH₂), 126.3, 128.8 (C-2, C-3, C-5, C-6), 129.7, 131.1, 144.6 (C-1, C-4, C-5'), 147.7 (C-4'), 151.4 (C-2'), 168.4, 169.0, 170.4 (CO); **LC-MS** (ESI) (90% H₂O to 100% MeOH in 10 min, then 100% MeOH to 15 min, DAD 220-400 nm), *t_R* = 9.13 min, 99% purity, *m/z* [M + H]⁺ calcd for C₂₉H₄₁N₇O₆S 616.29, found 616.4.

(2*S*,4*R*)-1-((*S*)-14-Azido-2-(*tert*-butyl)-4-oxo-6,9,12-trioxa-3-azatetradecanoyl)-4-hydroxy-*N*-((*S*)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)pyrrolidine-2-carboxamide (111)³²⁵

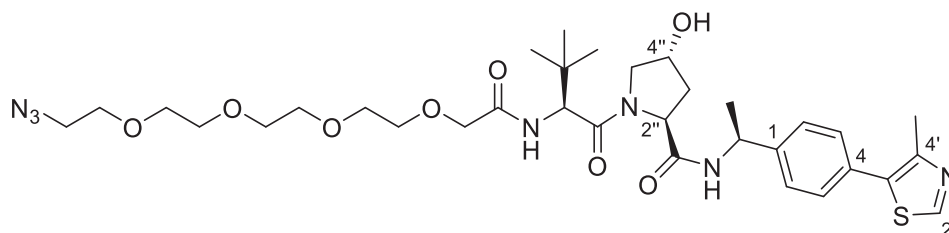


The Boc-protected VHL ligand **26** (872 mg, 1.60 mmol) was dissolved in dry CH₂Cl₂ (15 mL) and was treated with TFA (5 mL). The mixture was stirred for 2 h at room temperature. The mixture was concentrated *in vacuo*. *tert*-Butyl 2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)acetate (**77**, 463 mg, 1.60 mmol) was dissolved in dry CH₂Cl₂ (15 mL) and was treated with TFA (5 mL). The mixture was stirred for 2 h at room temperature. The mixture was concentrated *in vacuo*. The deprotected linker was dissolved in dry DMF (5 mL). HATU (669 mg, 1.76 mmol) and DIPEA (724 mg, 5.60 mmol) were added under argon. The mixture was allowed to stir for 30 min. The deprotected VHL ligand **96** was dissolved in dry DMF (5 mL) and was added to the mixture, containing the activated acid compound. The reaction mixture was allowed to stir at room temperature under argon for 4 h. The reaction was quenched by adding H₂O (25 mL) and was extracted with EtOAc (3 × 50 mL). The combined organic layers were washed with brine (75 mL), dried over Na₂SO₄, filtered and concentrated *in vacuo*. The crude product was purified by silica gel column chromatography using CH₂Cl₂/MeOH (19+1) as eluent to yield a slightly yellow resin (794 mg).

Yield 75%; **¹H NMR** (500 MHz, DMSO-*d*₆) δ 0.95 (s, 9H, C(CH₃)₃), 1.38 (d, *J* = 7.0 Hz, 3H, CHCH₃), 1.74 – 1.82 (m, 1H), 2.01 – 2.08 (m, 1H, 3''-H), 2.45 (s, 3H, CH₃ thiazole), 3.38 – 3.41 (m, 2H), 3.57 – 3.63 (m, 12H, 5''-H, CH₂), 3.96 (s, 2H, CH₂CON), 4.26 – 4.31 (m, 1H), 4.45 (t, *J* = 8.3 Hz, 1H), 4.55 (d, *J* = 9.5 Hz, 1H, 2''-H, 4''-H, NHCH), 4.87 – 4.94 (m, 1H, CHCH₃), 5.10 (d, *J* = 3.6 Hz, 1H, OH), 7.33 – 7.39 (m, 3H), 7.41 – 7.46 (m, 2H, 2-H, 3-H, 5-H, 6-H, CONH), 8.40 (d, *J* = 7.7 Hz, 1H, CONH), 8.97 (s, 1H, 2'-H); **¹³C NMR** (126 MHz, DMSO-*d*₆)

δ 15.9 (CH_3 thiazole), 22.4 (CHCH_3), 26.2 ($\text{C}(\text{CH}_3)_3$), 35.7 ($\text{C}(\text{CH}_3)_3$), 37.7 (C-3''), 47.7 (CHCH_3), 50.0 (CH_2N_3), 55.7, 56.5, 58.5 (C-2'' , C-5'' , NHCH), 68.7 (C-4''), 69.2, 69.6, 69.9, 70.4 (OCH_2), 126.3, 128.8 (C-2 , C-3 , C-5 , C-6), 129.7, 131.1, 144.6 (C-1 , C-4 , C-5'), 147.7 (C-4'), 151.4 (C-2'), 168.5, 169.0, 170.4 (CO), one signal (OCH_2) is missing due to overlapping signals; **LC-MS** (ESI) (90% H_2O to 100% MeOH in 10 min, then 100% MeOH to 15 min, DAD 220-400 nm), t_R = 9.24 min, 99% purity, m/z $[\text{M} + \text{H}]^+$ calcd for $\text{C}_{31}\text{H}_{45}\text{N}_7\text{O}_7\text{S}$ 660.32, found 660.5.

(2*S*,4*R*)-1-((*S*)-17-Azido-2-(*tert*-butyl)-4-oxo-6,9,12,15-tetraoxa-3-azaheptadecanoyl)-4-hydroxy-*N*-((*S*)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)pyrrolidine-2-carboxamide (112)

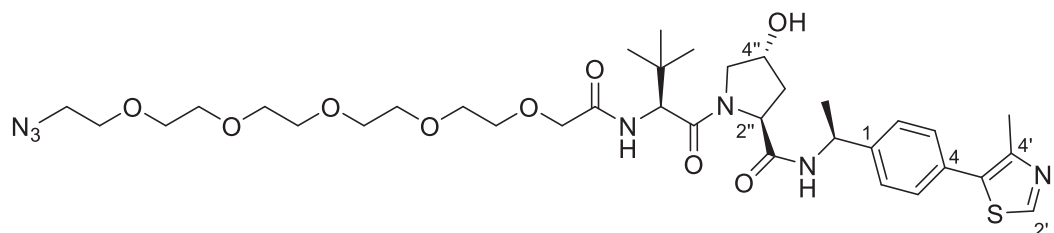


The Boc-protected VHL ligand **26** (545 mg, 1.00 mmol) was dissolved in dry CH_2Cl_2 (10 mL) and was treated with TFA (5 mL). The mixture was stirred for 2 h at room temperature. The mixture was concentrated *in vacuo*. *tert*-Butyl 14-azido-3,6,9,12-tetraoxatetradecanoate (**80**, 333 mg, 1.00 mmol) was dissolved in dry CH_2Cl_2 (10 mL) and was treated with TFA (5 mL). The mixture was stirred for 2 h at room temperature. The mixture was concentrated *in vacuo*. The deprotected linker was dissolved in dry DMF (5 mL). HATU (418 mg, 1.10 mmol) and DIPEA (452 mg, 3.50 mmol) were added under argon. The deprotected VHL ligand **96** was dissolved in dry DMF (5 mL) and was added to the mixture, containing the activated acid compound. The reaction mixture was allowed to stir at room temperature under argon for 18 h. The mixture was concentrated *in vacuo*. The crude product was purified by silica gel column chromatography using $\text{CH}_2\text{Cl}_2/\text{MeOH}$ (19+1) as eluent to yield a slightly yellow resin (507 mg).

Yield 72%; **^1H NMR** (600 MHz, $\text{DMSO-}d_6$) δ 0.95 (s, 9H, $\text{C}(\text{CH}_3)_3$), 1.38 (d, J = 7.0 Hz, 3H, CHCH_3), 1.74 – 1.82 (m, 1H), 2.01 – 2.08 (m, 1H, 3''-H), 2.45 (s, 3H, CH_3 thiazole), 3.37 – 3.40 (m, 2H), 3.53 – 3.63 (m, 16H, 5''-H, CH_2), 3.96 (s, 2H, CH_2CON), 4.26 – 4.30 (m, 1H), 4.45 (t, J = 8.1 Hz, 1H), 4.55 (d, J = 9.6 Hz, 1H, 2''-H, 4''-H, NHCH), 4.87 – 4.94 (m, 1H, CHCH_3), 5.11 (d, J = 3.5 Hz, 1H, OH), 7.33 – 7.39 (m, 3H), 7.41 – 7.46 (m, 2H, 2-H, 3-H, 5-H, 6-H, CONH), 8.41 (d, J = 7.7 Hz, 1H, CONH), 8.98 (s, 1H, 2'-H); **^{13}C NMR** (151 MHz, $\text{DMSO-}d_6$)

δ 15.9 (CH_3 thiazole), 22.4 (CHCH_3), 26.2 ($\text{C}(\text{CH}_3)_3$), 35.7 ($\text{C}(\text{CH}_3)_3$), 37.7 (C-3''), 47.7 (CHCH_3), 50.0 (CH_2N_3), 55.7, 56.5, 58.5 (C-2'', C-5'', NHCH), 68.7 (C-4''), 69.2, 69.6, 69.6, 69.7, 69.8, 69.8, 70.4 (OCH_2), 126.3, 128.8 (C-2, C-3, C-5, C-6), 129.7, 131.1, 144.7 (C-1, C-4, C-5'), 147.7 (C-4'), 151.4 (C-2'), 168.5, 169.0, 170.4 (CO), one signal (OCH_2) is missing due to overlapping signals; **LC-MS** (ESI) (90% H_2O to 100% MeCN in 10 min, then 100% MeCN to 15 min, DAD 200-600 nm), t_R = 5.99 min, 97% purity, m/z $[\text{M} + \text{H}]^+$ calcd for $\text{C}_{33}\text{H}_{49}\text{N}_7\text{O}_8\text{S}$ 704.34, found 704.6.

(2*S*,4*R*)-1-((*S*)-20-Azido-2-(*tert*-butyl)-4-oxo-6,9,12,15,18-pentaoxa-3-azaicosanoyl)-4-hydroxy-*N*-((*S*)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)pyrrolidine-2-carboxamide (113)

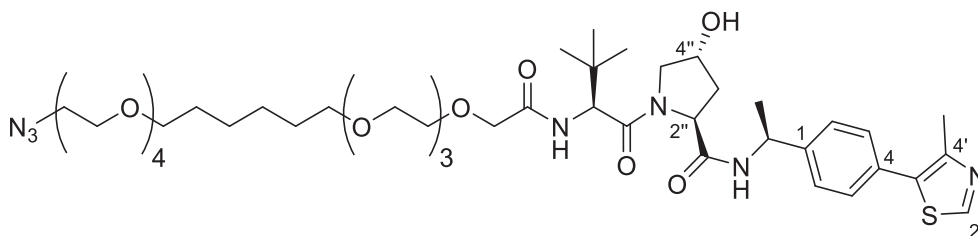


The Boc-protected VHL ligand **26** (599 mg, 1.10 mmol) was dissolved in dry CH_2Cl_2 (10 mL) and was treated with TFA (5 mL). The mixture was stirred for 2 h at room temperature. The mixture was concentrated *in vacuo*. *tert*-Butyl 17-azido-3,6,9,12,15-pentaoxaheptadecanoate (**83**, 415 mg, 1.10 mmol) was dissolved in dry CH_2Cl_2 (10 mL) and was treated with TFA (5 mL). The mixture was stirred for 2 h at room temperature. The mixture was concentrated *in vacuo*. The deprotected linker was dissolved in dry DMF (5 mL). HATU (460 mg, 1.21 mmol) and DIPEA (498 mg, 3.85 mmol) were added under argon. The deprotected VHL ligand **96** was dissolved in dry DMF (5 mL) and was added to the mixture, containing the activated acid compound. The reaction mixture was allowed to stir at room temperature under argon for 18 h. The mixture was concentrated *in vacuo*. The crude product was purified by silica gel column chromatography using $\text{CH}_2\text{Cl}_2/\text{MeOH}$ (19+1) as eluent to yield a slightly yellow resin (598 mg).

Yield 73%; **^1H NMR** (600 MHz, $\text{DMSO}-d_6$) δ 0.94 (s, 9H, $\text{C}(\text{CH}_3)_3$), 1.38 (d, J = 7.0 Hz, 3H, CHCH_3), 1.74 – 1.82 (m, 1H), 2.01 – 2.08 (m, 1H, 3''-H), 2.45 (s, 3H, CH_3 thiazole), 3.37 – 3.40 (m, 2H), 3.52 – 3.56 (m, 12H), 3.57 – 3.63 (m, 8H, 5''-H, CH_2), 3.96 (s, 2H, CH_2CON), 4.27 – 4.30 (m, 1H), 4.45 (t, J = 8.2 Hz, 1H), 4.55 (d, J = 9.5 Hz, 1H, 2''-H, 4''-H, NHCH), 4.88 – 4.94 (m, 1H, CHCH_3), 5.12 (d, J = 3.5 Hz, 1H, OH), 7.33 – 7.40 (m, 3H), 7.41 – 7.46 (m, 2H, 2-H, 3-H, 5-H, 6-H, CONH), 8.41 (d, J = 7.7 Hz, 1H, CONH), 8.98 (s, 1H, 2'-H); **^{13}C NMR**

(151 MHz, DMSO-*d*₆) δ 16.0 (CH₃ thiazole), 22.4 (CHCH₃), 26.2 (C(CH₃)₃), 35.7 (C(CH₃)₃), 37.7 (C-3''), 47.7 (CHCH₃), 50.0 (CH₂N₃), 55.7, 56.5, 58.5 (C-2'', C-5'', NHCH), 68.8 (C-4''), 69.2, 69.6, 69.6, 69.7, 69.8, 69.8, 69.8, 69.8, 70.4 (OCH₂), 126.3, 128.8 (C-2, C-3, C-5, C-6), 129.7, 131.1, 144.7 (C-1, C-4, C-5'), 147.7 (C-4'), 151.4 (C-2'), 168.5, 169.0, 170.4 (CO), one signal (OCH₂) is missing due to overlapping signals; **LC-MS** (ESI) (90% H₂O to 100% MeCN in 10 min, then 100% MeCN to 15 min, DAD 200-600 nm), *t*_R = 6.01 min, 100% purity, *m/z* [M + H]⁺ calcd for C₃₅H₅₃N₇O₉S 748.37, found 748.6.

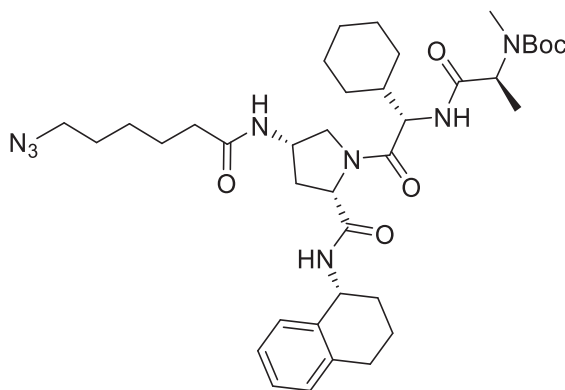
(2*S*,4*R*)-1-((*S*)-33-Azido-2-(*tert*-butyl)-4-oxo-6,9,12,15,22,25,28,31-octaoxa-3-azatritriacontanoyl)-4-hydroxy-*N*-((*S*)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)pyrrolidine-2-carboxamide (114)



The Boc-protected VHL ligand **26** (654 mg, 1.20 mmol) was dissolved in dry CH₂Cl₂ (15 mL) and was treated with TFA (5 mL). The mixture was stirred for 2 h at room temperature. The mixture was concentrated *in vacuo*. 30-Azido-3,6,9,12,19,22,25,28-octaoxatriacontanoic acid (**86**, 612 mg, 1.20 mmol) was dissolved in dry DMF (5 mL). HATU (502 mg, 1.32 mmol) and DIPEA (543 mg, 4.20 mmol) were added under argon. The deprotected VHL ligand **96** was dissolved in dry DMF (5 mL) and was added to the mixture, containing the activated acid compound. The reaction mixture was allowed to stir at room temperature under argon for 4 h. The mixture was concentrated *in vacuo*. The crude product was purified by silica gel column chromatography using CH₂Cl₂/MeOH (19+1) as eluent to yield a slightly yellow resin (368 mg).

Yield 33%; ¹H NMR (500 MHz, DMSO-*d*₆) δ 0.95 (s, 9H, C(CH₃)₃), 1.25 – 1.32 (m, 4H, CH₂), 1.38 (d, *J* = 7.0 Hz, 3H, CHCH₃), 1.44 – 1.51 (m, 4H, CH₂), 1.75 – 1.83 (m, 1H), 2.01 – 2.08 (m, 1H, 3''-H), 2.46 (s, 3H, CH₃ thiazole), 3.33 – 3.41 (m, 6H), 3.44 – 3.48 (m, 4H), 3.48 – 3.57 (m, 16H), 3.57 – 3.63 (m, 8H, 5''-H, CH₂), 3.96 (s, 2H, CH₂CON), 4.27 – 4.31 (m, 1H), 4.45 (t, *J* = 8.2 Hz, 1H), 4.55 (d, *J* = 9.5 Hz, 1H, 2''-H, 4''-H, NHCH), 4.88 – 4.95 (m, 1H, CHCH₃), 5.10 (d, *J* = 3.5 Hz, 1H, OH), 7.33 – 7.40 (m, 3H), 7.41 – 7.46 (m, 2H, 2-H, 3-H, 5-H, 6-H, CONH), 8.39 (d, *J* = 7.7 Hz, 1H, CONH), 8.97 (s, 1H, 2'-H); ¹³C NMR (126 MHz, DMSO-*d*₆)

***tert*-Butyl ((*S*)-1-(((*S*)-2-((2*S*,4*S*)-4-(6-azidohexanamido)-2-(((*R*)-1,2,3,4-tetrahydronaphthalen-1-yl)carbamoyl)pyrrolidin-1-yl)-1-cyclohexyl-2-oxoethyl)amino)-1-oxopropan-2-yl)(methyl)carbamate (115)**

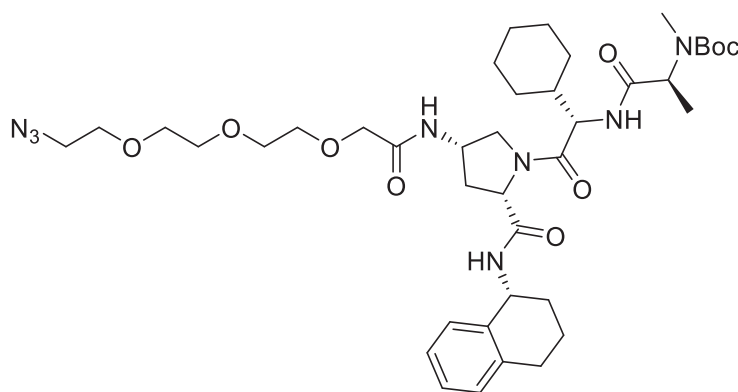


6-Azidohexanoic acid (**69**, 157 mg, 1.00 mmol) was dissolved in dry DMF (5 mL). HATU (418 mg, 1.10 mmol) and DIPEA (452 mg, 3.5 mmol) were added under argon. The IAP ligand **32** (584 mg, 1.00 mmol) was dissolved in dry DMF (5 mL) and was added to the mixture, containing the activated acid compound. The reaction mixture was stirred at room temperature under argon for 18 h. The reaction mixture was concentrated *in vacuo*. The residue was diluted with saturated NH₄Cl solution (50 mL) and was extracted with CH₂Cl₂ (2 × 50 mL). The combined organic layer was washed brine (50 mL), dried over Na₂SO₄, filtered and concentrated *in vacuo*. The crude product was purified by silica gel flash column chromatography using a CH₂Cl₂/MeOH gradient (0% to 5% MeOH) as eluent to yield a white solid (565 mg).

Yield 78%; mp: 50-56 °C; **¹H NMR** (500 MHz, DMSO-*d*₆) δ 0.89 – 1.04 (m, 2H), 1.07 – 1.23 (m, 6H), 1.28 – 1.34 (m, 2H), 1.41 (s, 9H), 1.49 – 1.56 (m, 4H), 1.57 – 1.78 (m, 8H), 1.81 – 1.90 (m, 3H), 2.07 (t, *J* = 7.4 Hz, 2H), 2.33 – 2.42 (m, 1H), 2.70 – 2.79 (m, 5H), 4.01 – 4.09 (m, 1H), 4.23 – 4.38 (m, 3H), 4.42 – 4.62 (m, 1H), 4.90 – 4.97 (m, 1H), 7.06 – 7.12 (m, 2H),

7.12 – 7.17 (m, 1H), 7.31 (d, $J = 7.4$ Hz, 1H), 7.61 – 7.89 (m, 1H), 8.13 (d, $J = 7.7$ Hz, 1H), 8.38 (d, $J = 8.6$ Hz, 1H), two overlapping signals (3H) are obscured by solvent signal at 3.30 ppm; ^{13}C NMR (126 MHz, DMSO- d_6) δ 20.2, 24.5, 25.5, 25.7, 25.8, 27.9, 28.0, 28.7, 29.7, 29.9, 34.3, 35.2, 46.6, 47.7, 50.5, 52.2, 54.8, 58.4, 78.9, 125.5, 126.5, 128.3, 128.4, 136.9, 137.3, 169.7, 170.8, 171.7; LC-MS (ESI) (90% H₂O to 100% MeCN in 10 min, then 100% MeCN to 15 min, DAD 220-600 nm), $t_R = 9.00$ min, 91% purity, m/z $[\text{M} + \text{H}]^+$ calcd for C₃₈H₅₈N₈O₆ 723.46, found 723.5.

***tert*-Butyl ((*S*)-1-(((*S*)-2-((2*S*,4*S*)-4-(2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)acetamido)-2-(((*R*)-1,2,3,4-tetrahydronaphthalen-1-yl)carbamoyl)pyrrolidin-1-yl)-1-cyclohexyl-2-oxoethyl)amino)-1-oxopropan-2-yl)(methyl)carbamate (116)**

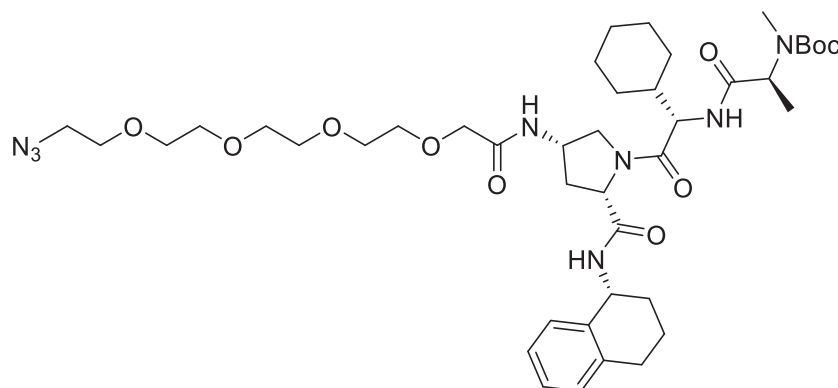


tert-Butyl 2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)acetate (**77**, 203 mg, 700 μmol) was dissolved in dry CH₂Cl₂ (5 mL) and was treated with TFA (5 mL). The mixture was stirred for 2 h at room temperature. The mixture was concentrated *in vacuo*. The deprotected linker was dissolved in dry DMF (5 mL). HATU (293 mg, 770 μmol) and DIPEA (317 mg, 2.45 mmol) were added under argon. The IAP ligand **32** (409 mg, 700 μmol) was dissolved in dry DMF (5 mL) and was added to the mixture, containing the activated acid compound. The reaction mixture was stirred at room temperature under argon for 18 h. The reaction mixture was concentrated *in vacuo*. The crude product was purified by silica gel flash column chromatography using a CH₂Cl₂/MeOH gradient (0% to 5% MeOH) as eluent to yield a colorless resin (468 mg).

Yield 84%; ^1H NMR (600 MHz, DMSO- d_6) δ 0.89 – 1.04 (m, 2H), 1.07 – 1.25 (m, 6H), 1.39 (s, 9H), 1.56 – 1.91 (m, 11H), 2.33 – 2.42 (m, 1H), 2.68 – 2.79 (m, 5H), 3.34 – 3.38 (m, 2H), 3.45 – 3.50 (m, 1H), 3.53 – 3.62 (m, 10H), 3.85 – 3.92 (m, 2H), 3.99 (dd, $J = 10.1, 6.4$ Hz, 1H), 4.27 – 4.39 (m, 2H), 4.40 – 4.62 (m, 2H), 4.89 – 4.98 (m, 1H), 7.05 – 7.12 (m, 2H), 7.12 – 7.17

(m, 1H), 7.31 (d, $J = 7.7$ Hz, 1H), 7.62 – 7.89 (m, 1H), 8.33 (d, $J = 8.4$ Hz, 1H), 8.44 (d, $J = 8.6$ Hz, 1H); ^{13}C NMR (151 MHz, DMSO- d_6) δ 20.2, 25.5, 25.8, 25.8, 28.0, 28.6, 28.7, 29.7, 30.0, 34.2, 46.7, 47.4, 50.0, 52.9, 54.8, 58.5, 69.2, 69.6, 69.8, 70.0, 70.4, 78.9, 125.6, 126.6, 128.3, 128.5, 136.9, 137.3, 169.0, 169.8, 171.1; LC-MS (ESI) (90% H₂O to 100% MeCN in 10 min, then 100% MeCN to 15 min, DAD 220-600 nm), $t_R = 8.22$ min, 92% purity, m/z $[\text{M} + \text{H}]^+$ calcd for C₄₀H₆₂N₈O₉ 799.47, found 799.8.

***tert*-Butyl ((*S*)-1-(((*S*)-2-((2*S*,4*S*)-4-(14-azido-3,6,9,12-tetraoxatetradecanamido)-2-(((*R*)-1,2,3,4-tetrahydronaphthalen-1-yl)carbamoyl)pyrrolidin-1-yl)-1-cyclohexyl-2-oxoethyl)amino)-1-oxopropan-2-yl)(methyl)carbamate (117)**

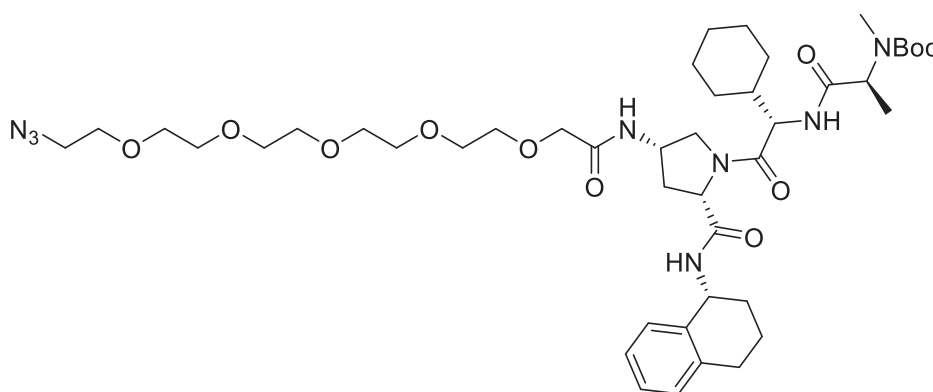


tert-Butyl 14-azido-3,6,9,12-tetraoxatetradecanoate (**80**, 400 mg, 1.20 mmol) was dissolved in dry CH₂Cl₂ (5 mL) and was treated with TFA (5 mL). The mixture was stirred for 2 h at room temperature. The mixture was concentrated *in vacuo*. The deprotected linker was dissolved in dry DMF (5 mL). HATU (502 mg, 1.32 mmol) and DIPEA (543 mg, 4.20 mmol) were added under argon. The IAP ligand **32** (701 mg, 1.20 mmol) was dissolved in dry DMF (5 mL) and was added to the mixture, containing the activated acid compound. The reaction mixture was stirred at room temperature under argon for 18 h. The reaction mixture was concentrated *in vacuo*. The crude product was purified by silica gel flash column chromatography using a CH₂Cl₂/MeOH gradient (0% to 5% MeOH) as eluent to yield a colorless resin (704 mg).

Yield 70%; ^1H NMR (600 MHz, DMSO- d_6) δ 0.89 – 1.04 (m, 2H), 1.07 – 1.25 (m, 6H), 1.41 (s, 9H), 1.57 – 1.90 (m, 11H), 2.33 – 2.42 (m, 1H), 2.68 – 2.79 (m, 5H), 3.35 – 3.39 (m, 2H), 3.45 – 3.49 (m, 1H), 3.49 – 3.63 (m, 14H), 3.84 – 3.93 (m, 2H), 3.99 (dd, $J = 10.1, 6.4$ Hz, 1H), 4.27 – 4.37 (m, 2H), 4.40 – 4.62 (m, 2H), 4.90 – 4.98 (m, 1H), 7.05 – 7.12 (m, 2H), 7.12 – 7.17 (m, 1H), 7.31 (d, $J = 7.6$ Hz, 1H), 7.58 – 7.90 (m, 1H), 8.34 (d, $J = 8.4$ Hz, 1H), 8.44 (d, $J = 8.7$ Hz, 1H); ^{13}C NMR (151 MHz, DMSO- d_6) δ 20.2, 25.5, 25.8, 25.8, 28.0, 28.6, 28.7, 29.7, 30.0,

34.2, 46.7, 47.4, 50.0, 52.9, 54.8, 58.5, 69.2, 69.6, 69.7, 69.8, 69.8, 69.8, 70.0, 70.4, 78.9, 125.6, 126.6, 128.3, 128.5, 136.9, 137.3, 169.0, 169.8, 171.1; **LC-MS** (ESI) (90% H₂O to 100% MeCN in 10 min, then 100% MeCN to 15 min, DAD 220-600 nm), t_R = 8.17 min, 90% purity, m/z [M + H]⁺ calcd for C₄₂H₆₆N₈O₁₀ 843.50, found 843.8.

***tert*-Butyl ((*S*)-1-(((*S*)-2-((2*S*,4*S*)-4-(17-azido-3,6,9,12,15-pentaoxaheptadecanamido)-2-(((*R*)-1,2,3,4-tetrahydronaphthalen-1-yl)carbamoyl)pyrrolidin-1-yl)-1-cyclohexyl-2-oxoethyl)amino)-1-oxopropan-2-yl)(methyl)carbamate (118)**

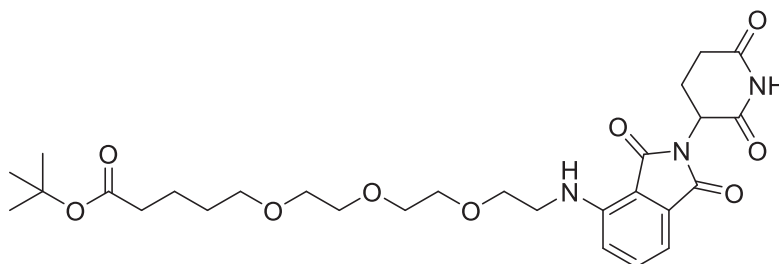


tert-Butyl 17-azido-3,6,9,12,15-pentaoxaheptadecanoate (**83**, 415 mg, 1.10 mmol) was dissolved in dry CH₂Cl₂ (5 mL) and was treated with TFA (5 mL). The mixture was stirred for 2 h at room temperature. The mixture was concentrated *in vacuo*. The deprotected linker was dissolved in dry DMF (5 mL). HATU (460 mg, 1.21 mmol) and DIPEA (498 mg, 3.85 mmol) were added under argon. The IAP ligand **32** (642 mg, 1.10 mmol) was dissolved in dry DMF (5 mL) and was added to the mixture, containing the activated acid compound. The reaction mixture was stirred at room temperature under argon for 18 h. The reaction mixture was concentrated *in vacuo*. The crude product was purified by silica gel flash column chromatography using a CH₂Cl₂/MeOH gradient (0% to 5% MeOH) as eluent to yield a colorless resin (598 mg).

Yield 61%; ¹H NMR (600 MHz, DMSO-*d*₆) δ 0.88 – 1.04 (m, 2H), 1.07 – 1.24 (m, 6H), 1.35 – 1.43 (m, 9H), 1.57 – 1.89 (m, 11H), 2.32 – 2.44 (m, 1H), 2.68 – 2.79 (m, 5H), 3.35 – 3.41 (m, 2H), 3.42 – 3.67 (m, 19H), 3.84 – 3.92 (m, 2H), 3.99 (dd, *J* = 10.2, 6.4 Hz, 1H), 4.26 – 4.37 (m, 2H), 4.38 – 4.61 (m, 2H), 4.89 – 4.97 (m, 1H), 7.06 – 7.12 (m, 2H), 7.12 – 7.17 (m, 1H), 7.25 – 7.40 (m, 1H), 7.58 – 7.88 (m, 1H), 8.34 (d, *J* = 8.4 Hz, 1H), 8.44 (d, *J* = 8.7 Hz, 1H); ¹³C NMR (151 MHz, DMSO-*d*₆) δ 20.2, 25.5, 25.8, 25.8, 28.0, 28.6, 28.7, 29.7, 30.0, 34.2, 46.7, 47.4, 50.0, 52.9, 54.8, 58.5, 69.2, 69.6, 69.7, 69.7, 69.7, 69.8, 69.8, 70.0, 70.4, 78.9, 125.6,

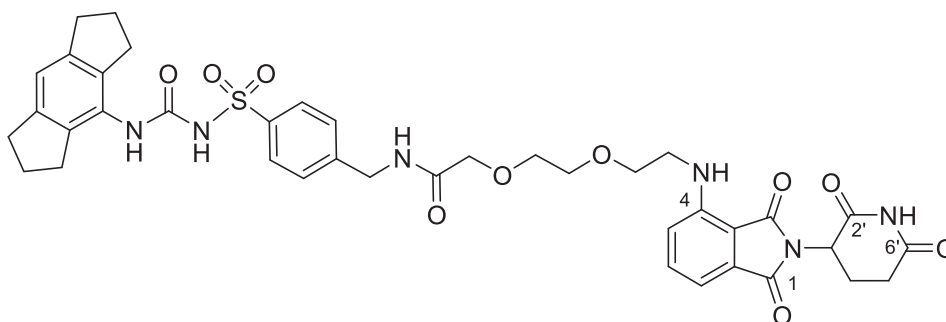
126.6, 128.3, 128.5, 136.9, 137.3, 169.0, 169.8, 171.1; **LC-MS** (ESI) (90% H₂O to 100% MeCN in 10 min, then 100% MeCN to 15 min, DAD 220-600 nm), t_R = 8.12 min, 93% purity, m/z [M + H]⁺ calcd for C₄₄H₇₀N₈O₁₁ 887.52, found 887.8.

tert-Butyl 5-(2-(2-(2-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisindolin-4-yl)amino)ethoxy)ethoxy)ethoxy)pentanoate (119)²⁴⁹



Compound **119** was synthesized and provided by Dr. Christian Steinebach, University of Bonn.

2-(2-(2-(2-((2-(2,6-Dioxopiperidin-3-yl)-1,3-dioxoisindolin-4-yl)amino)ethoxy)ethoxy)-N-(4-(N-((1,2,3,5,6,7-hexahydro-s-indacen-4-yl)carbamoyl)sulfamoyl)benzyl)acetamide (120)

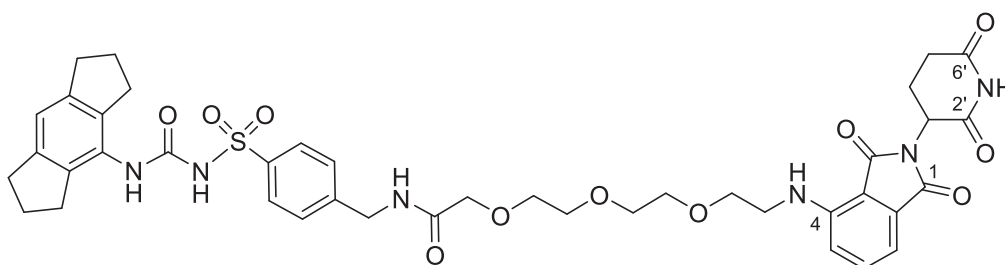


Compound **87** (143 mg, 300 μ mol) was dissolved in dry CH₂Cl₂ (4 mL), treated with TFA (4 mL) and stirred at room temperature for 2 h. The mixture was concentrated *in vacuo*. The NLRP3 ligand **5** (146 mg, 300 μ mol) was dissolved in dry CH₂Cl₂ (4 mL), treated with TFA (4 mL) and stirred at room temperature for 2 h. The mixture was concentrated *in vacuo*. The deprotected acid compound was dissolved in dry DMF (8 mL). DIPEA (116 mg, 900 μ mol) and HATU (125 mg, 330 μ mol) were added under argon. The deprotected NLRP3 ligand was dissolved in dry DMF (8 mL) and was added to the mixture, containing the activated acid compound. The reaction mixture was stirred for 2 h at room temperature under argon. The mixture was concentrated *in vacuo*. The crude product was purified by silica gel flash column

chromatography using a CH₂Cl₂/MeOH gradient (0% to 20% MeOH) to yield a yellow solid (123 mg).

Yield 52%; mp: 137-143 °C; ¹H NMR (600 MHz, DMSO-*d*₆) δ 1.91 (quint, *J* = 7.4 Hz, 4H, CH₂ indacene), 1.98 – 2.04 (m, 1H, 4'-H), 2.50 – 2.62 (m, 6H, 4'-H, 5'-H, CH₂ indacene), 2.76 (t, *J* = 7.4 Hz, 4H, CH₂ indacene), 2.83 – 2.90 (m, 1H, 5'-H), 3.44 (q, *J* = 5.6 Hz, 2H), 3.60 – 3.64 (m, 6H), 3.97 (s, 2H, OCH₂, CH₂NH), 4.37 (d, *J* = 6.2 Hz, 2H, CH₂NHCO), 5.04 (dd, *J* = 12.8, 5.5 Hz, 1H, 3'-H), 6.59 (t, *J* = 5.9 Hz, 1H, NHCH₂), 6.91 (s, 1H, CH_{arom.} indacene), 7.03 (d, *J* = 7.0 Hz, 1H), 7.11 (d, *J* = 8.6 Hz, 1H, 5-H, 7-H), 7.44 – 7.47 (m, 2H, CH_{arom.}), 7.56 (dd, *J* = 8.5, 7.1 Hz, 1H, 6-H), 7.85 – 7.88 (m, 2H, CH_{arom.}), 8.06 (s, 1H, NHCON), 8.33 (t, *J* = 6.3 Hz, 1H, CH₂NHCO), 10.71 (s, 1H, SO₂NH), 11.07 (s, 1H, NH imide); ¹³C NMR (151 MHz, DMSO-*d*₆) δ 22.1, 25.0, 30.1, 31.0, 32.4, 41.3, 41.7, 48.5, 68.9, 69.4, 70.0, 70.3, 109.3, 110.7, 117.4, 117.9, 127.2, 127.5, 128.6, 132.1, 136.2, 137.1, 138.5, 143.0, 145.1, 146.3, 149.1, 167.2, 168.9, 169.5, 170.0, 172.7; LC-MS (ESI) (90% H₂O to 100% MeOH in 10 min, then 100% MeOH to 20 min, DAD 220-500 nm), *t*_R = 10.72 min, 94% purity, *m/z* [M + H]⁺ calcd for C₃₉H₄₂N₆O₁₀S 787.28, found 787.7; HRMS (ESI) *m/z* [M + Na]⁺ calcd for C₃₉H₄₂N₆O₁₀S 809.2575, found 809.2561.

2-(2-(2-(2-((2-(2,6-Dioxopiperidin-3-yl)-1,3-dioxoisindolin-4-yl)amino)ethoxy)ethoxy)ethoxy)-N-(4-(N-((1,2,3,5,6,7-hexahydro-s-indacen-4-yl)carbamoyl)sulfamoyl)benzyl)acetamide (121)

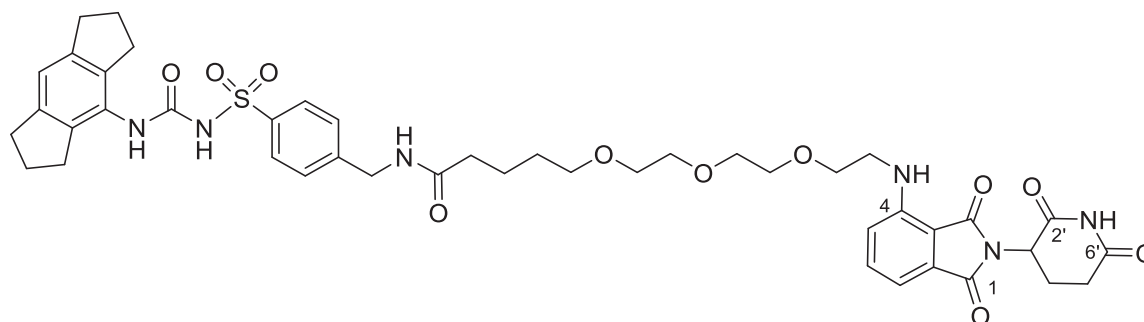


Compound **88** (197 mg, 380 μmol) was dissolved in dry CH₂Cl₂ (5 mL), treated with TFA (5 mL) and stirred at room temperature for 2 h. The mixture was concentrated *in vacuo*. The NLRP3 ligand **5** (185 mg, 380 μmol) was dissolved in dry CH₂Cl₂ (5 mL), treated with TFA (5 mL) and stirred at room temperature for 2 h. The mixture was concentrated *in vacuo*. The deprotected acid compound was dissolved in dry DMF (4 mL). DIPEA (147 mg, 1.14 mmol) and HATU (160 mg, 420 μmol) were added under argon. The deprotected NLRP3 ligand was dissolved in dry DMF (4 mL) and was added to the mixture, containing the activated acid compound. The reaction mixture was stirred for 2 h at room temperature under argon. The mixture was concentrated *in vacuo*. The crude product was purified by silica gel flash column

chromatography using a CH₂Cl₂/MeOH gradient (0% to 20% MeOH) to yield a yellow solid (92 mg).

Yield 29%; mp: 130-136 °C; ¹H NMR (600 MHz, DMSO-*d*₆) δ 1.91 (quint, *J* = 7.4 Hz, 4H, CH₂ indacene), 1.98 – 2.05 (m, 1H, 4'-H), 2.51 – 2.61 (m, 6H, 4'-H, 5'-H, CH₂ indacene), 2.76 (t, *J* = 7.4 Hz, 4H, CH₂ indacene), 2.82 – 2.91 (m, 1H, 5'-H), 3.43 (q, *J* = 5.6 Hz, 2H), 3.52 – 3.54 (m, 4H), 3.56 – 3.61 (m, 6H), 3.95 (s, 2H, OCH₂, CH₂NH), 4.38 (d, *J* = 6.2 Hz, 2H, CH₂NHCO), 5.04 (dd, *J* = 12.8, 5.4 Hz, 1H, 3'-H), 6.58 (t, *J* = 5.9 Hz, 1H, NHCH₂), 6.90 (s, 1H, CH_{arom.} indacene), 7.03 (d, *J* = 7.0 Hz, 1H), 7.11 (d, *J* = 8.6 Hz, 1H, 5-H, 7-H), 7.44 – 7.48 (m, 2H, CH_{arom.}), 7.56 (dd, *J* = 8.4, 7.2 Hz, 1H, 6-H), 7.84 – 7.89 (m, 2H, CH_{arom.}), 8.05 (s, 1H, NHCON), 8.33 (t, *J* = 6.3 Hz, 1H, CH₂NHCO), 10.73 (s, 1H, SO₂NH), 11.07 (s, 1H, NH imide); ¹³C NMR (151 MHz, DMSO-*d*₆) δ 22.1, 25.0, 30.1, 30.9, 32.4, 41.3, 41.6, 48.5, 68.8, 69.6, 69.7, 69.7, 70.0, 70.3, 109.2, 110.6, 117.4, 117.9, 127.2, 127.5, 128.7, 132.1, 136.2, 137.1, 138.6, 143.0, 145.0, 146.4, 149.2, 167.3, 168.9, 169.6, 170.0, 172.7; LC-MS (ESI) (90% H₂O to 100% MeOH in 10 min, then 100% MeOH to 20 min, DAD 220-500 nm), *t*_R = 10.83 min, 98% purity, *m/z* [M + H]⁺ calcd for C₄₁H₄₆N₆O₁₁S 831.30, found 831.6; HRMS (ESI) *m/z* [M + Na]⁺ calcd for C₄₁H₄₆N₆O₁₁S 853.2837, found 853.2830.

5-(2-(2-(2-((2-(2,6-Dioxopiperidin-3-yl)-1,3-dioxoisindolin-4-yl)amino)ethoxy)ethoxy)ethoxy)-*N*-(4-(*N*-((1,2,3,5,6,7-hexahydro-*s*-indacen-4-yl)carbamoyl)sulfamoyl)benzyl)pentanamide (122)

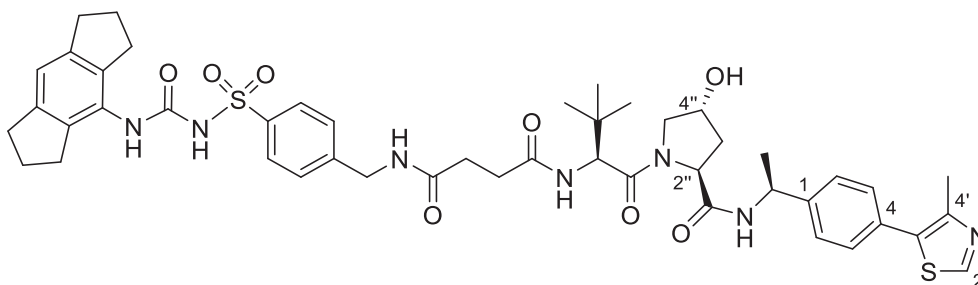


Compound **119** (168 mg, 300 μmol) was dissolved in dry CH₂Cl₂ (4 mL), treated with TFA (4 mL) and stirred at room temperature for 2 h. The mixture was concentrated *in vacuo*. The NLRP3 ligand **5** (146 mg, 300 μmol) was dissolved in dry CH₂Cl₂ (4 mL), treated with TFA (4 mL) and stirred at room temperature for 2 h. The mixture was concentrated *in vacuo*. The deprotected acid compound was dissolved in dry DMF (5 mL). DIPEA (116 mg, 900 μmol) and HATU (125 mg, 330 μmol) were added under argon. The deprotected NLRP3 ligand was dissolved in dry DMF (5 mL) and was added to the mixture, containing the activated acid

compound. The reaction mixture was stirred for 2 h at room temperature under argon. The mixture was concentrated *in vacuo*. The crude product was purified by silica gel flash column chromatography using a CH₂Cl₂/MeOH gradient (0% to 20% MeOH) to yield a yellow solid (134 mg).

Yield 51%; mp: 118-124 °C; ¹H NMR (600 MHz, DMSO-*d*₆) δ 1.43 – 1.50 (m, 2H), 1.51 – 1.59 (m, 2H, CH₂), 1.91 (quint, *J* = 7.4 Hz, 4H, CH₂ indacene), 1.99 – 2.05 (m, 1H, 4'-H), 2.15 (t, *J* = 7.4 Hz, 2H, CH₂), 2.51 – 2.62 (m, 6H, 4'-H, 5'-H, CH₂ indacene), 2.76 (t, *J* = 7.4 Hz, 4H, CH₂ indacene), 2.84 – 2.94 (m, 1H, 5'-H), 3.35 (t, *J* = 6.4 Hz, 2H), 3.42 – 3.48 (m, 4H), 3.49 – 3.51 (m, 2H), 3.51 – 3.54 (m, 2H), 3.54 – 3.57 (m, 2H), 3.62 (t, *J* = 5.5 Hz, 2H, OCH₂, CH₂NH), 4.33 (d, *J* = 5.9 Hz, 2H, CH₂NHCO), 5.05 (dd, *J* = 12.8, 5.4 Hz, 1H, 3'-H), 6.59 (t, *J* = 5.9 Hz, 1H, NHCH₂), 6.91 (s, 1H, CH_{arom.} indacene), 7.03 (d, *J* = 7.0 Hz, 1H), 7.13 (d, *J* = 8.6 Hz, 1H, 5-H, 7-H), 7.41 – 7.47 (m, 2H, CH_{arom.}), 7.57 (dd, *J* = 8.5, 7.0 Hz, 1H, 6-H), 7.85 – 7.89 (m, 2H, CH_{arom.}), 8.06 (s, 1H, NHCON), 8.37 (t, *J* = 6.0 Hz, 1H, CH₂NHCO), 10.71 (s, 1H, SO₂NH), 11.07 (s, 1H, NH imide); ¹³C NMR (151 MHz, DMSO-*d*₆) δ 22.0, 22.1, 25.0, 28.8, 30.1, 31.0, 32.4, 35.0, 41.6, 41.7, 48.5, 68.9, 69.4, 69.7, 69.8, 69.8, 69.9, 109.2, 110.6, 117.4, 117.9, 127.3, 127.4, 128.6, 132.1, 136.2, 137.1, 138.5, 143.0, 145.4, 146.4, 149.1, 167.3, 168.9, 170.0, 172.2, 172.7; LC-MS (ESI) (90% H₂O to 100% MeOH in 10 min, then 100% MeOH to 20 min, DAD 220-500 nm), *t*_R = 10.97 min, 98% purity, *m/z* [M + H]⁺ calcd for C₄₄H₅₂N₆O₁₁S 873.35, found 873.6; HRMS (ESI) *m/z* [M + Na]⁺ calcd for C₄₄H₅₂N₆O₁₁S 895.3307, found 895.3306.

*N*¹-(4-(*N*-((1,2,3,5,6,7-Hexahydro-*s*-indacen-4-yl)carbamoyl)sulfamoyl)benzyl)-*N*⁴-((*S*)-1-((2*S*,4*R*)-4-hydroxy-2-(((*S*)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)carbamoyl)pyrrolidin-1-yl)-3,3-dimethyl-1-oxobutan-2-yl)succinamide (123)

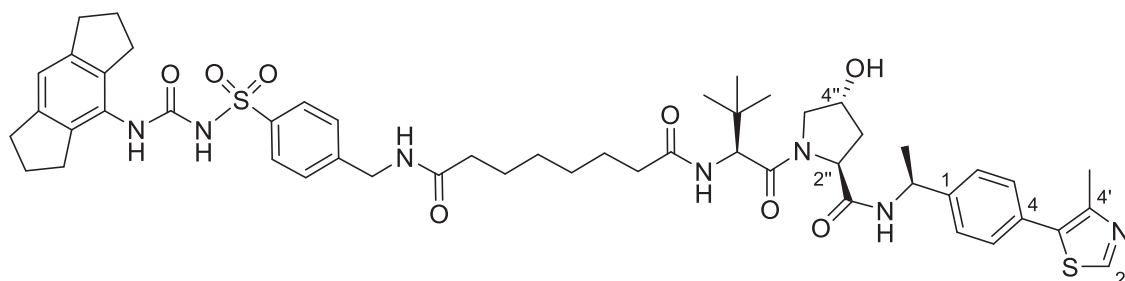


Compound **98** (180 mg, 300 μmol) was dissolved in dry CH₂Cl₂ (3 mL), treated with TFA (3 mL) and stirred at room temperature for 2 h. The mixture was concentrated *in vacuo*. The NLRP3 ligand **5** (146 mg, 300 μmol) was dissolved in dry CH₂Cl₂ (3 mL), treated with TFA (3

mL) and stirred at room temperature for 2 h. The mixture was concentrated *in vacuo*. The deprotected acid compound was dissolved in dry DMF (6 mL). DIPEA (116 mg, 900 μ mol) and HATU (125 mg, 330 μ mol) were added under argon. The deprotected NLRP3 ligand was dissolved in dry DMF (6 mL) and was added to the mixture, containing the activated acid compound. The reaction mixture was stirred for 18 h at room temperature under argon. The mixture was concentrated *in vacuo*. The residue was diluted with saturated NH_4Cl solution (50 mL) and was extracted with EtOAc (3×30 mL). The combined organic layers were washed with saturated NH_4Cl solution (60 mL), dried over Na_2SO_4 , filtered and concentrated *in vacuo*. The crude product was purified by silica gel column chromatography using $\text{CH}_2\text{Cl}_2/\text{MeOH}$ (9+1) as eluent to yield a white solid (23 mg).

Yield 8%; mp: 133-139 $^\circ\text{C}$; ^1H NMR (600 MHz, $\text{DMSO}-d_6$) δ 0.93 (s, 9H, $\text{C}(\text{CH}_3)_3$), 1.37 (d, $J = 7.0$ Hz, 3H, CHCH_3), 1.76 – 1.81 (m, 1H, 3''-H), 1.92 (quint, $J = 7.5$ Hz, 4H, CH_2 indacene), 1.98 – 2.04 (m, 1H, 3''-H), 2.31 – 2.45 (m, 4H, CH_2), 2.45 (s, 3H, CH_3 thiazole), 2.54 (t, $J = 7.4$ Hz, 4H, CH_2 indacene), 2.77 (t, $J = 7.4$ Hz, 4H, CH_2 indacene), 3.55 – 3.64 (m, 2H, 5''-H), 4.24 – 4.29 (m, 1H), 4.31 – 4.36 (m, 2H), 4.43 (t, $J = 8.0$ Hz, 1H), 4.51 (d, $J = 9.3$ Hz, 1H, 2''-H, 4''-H, NHCH , CH_2NHCO), 4.87 – 4.95 (m, 1H, CHCH_3), 5.07 (d, $J = 3.6$ Hz, 1H, OH), 6.92 (s, 1H, $\text{CH}_{\text{arom. indacene}}$), 7.36 – 7.40 (m, 2H), 7.42 – 7.48 (m, 4H), 7.83 – 7.89 (m, 3H, $\text{CH}_{\text{arom. CONH}}$), 8.15 (s, 1H, NHCON), 8.35 (d, $J = 7.8$ Hz, 1H, CONH), 8.43 (t, $J = 6.1$ Hz, 1H, CH_2NHCO), 8.98 (s, 1H, 2'-H), 10.76 (s, 1H, SO_2NH); ^{13}C NMR (151 MHz, $\text{DMSO}-d_6$) δ 15.9, 22.4, 25.0, 26.4, 30.1, 30.5, 30.9, 32.4, 35.3, 37.7, 41.7, 47.7, 56.2, 56.5, 58.5, 68.7, 117.9, 126.3, 127.2, 127.4, 128.6, 128.8, 129.7, 131.1, 137.1, 138.4, 143.0, 144.6, 145.4, 147.7, 149.0, 151.4, 169.4, 170.6, 171.1, 171.7; LC-MS (ESI) (90% H_2O to 100% MeOH in 10 min, then 100% MeOH to 20 min, DAD 220-400 nm), $t_R = 11.39$ min, 95% purity, m/z $[\text{M} + \text{H}]^+$ calcd for $\text{C}_{47}\text{H}_{57}\text{N}_7\text{O}_8\text{S}_2$ 912.38, found 912.4; HRMS (ESI) m/z $[\text{M} + \text{Na}]^+$ calcd for $\text{C}_{47}\text{H}_{57}\text{N}_7\text{O}_8\text{S}_2$ 934.3602, found 934.3626.

***N*¹-(4-(*N*-((1,2,3,5,6,7-Hexahydro-*s*-indacen-4-yl)carbamoyl)sulfamoyl)benzyl)-*N*⁸-((*S*)-1-((2*S*,4*R*)-4-hydroxy-2-(((*S*)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)carbamoyl)pyrrolidin-1-yl)-3,3-dimethyl-1-oxobutan-2-yl)octanediamide (124)**

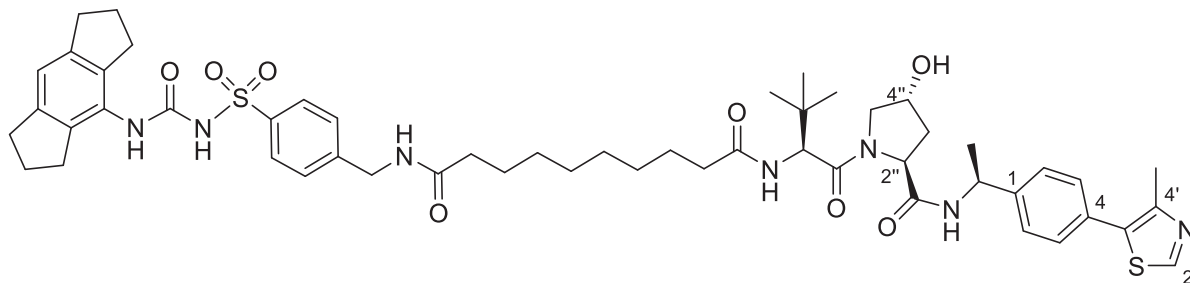


Compound **100** (197 mg, 300 μ mol) was dissolved in dry CH_2Cl_2 (3 mL), treated with TFA (3 mL) and stirred at room temperature for 2 h. The mixture was concentrated *in vacuo*. The NLRP3 ligand **5** (146 mg, 300 μ mol) was dissolved in dry CH_2Cl_2 (3 mL), treated with TFA (3 mL) and stirred at room temperature for 2 h. The mixture was concentrated *in vacuo*. The deprotected acid compound was dissolved in dry DMF (6 mL). DIPEA (116 mg, 900 μ mol) and HATU (125 mg, 330 μ mol) were added under argon. The deprotected NLRP3 ligand was dissolved in dry DMF (6 mL) and was added to the mixture, containing the activated acid compound. The reaction mixture was stirred for 4 h at room temperature under argon. The mixture was concentrated *in vacuo*. The residue was diluted with saturated NH_4Cl solution (50 mL) and was extracted with EtOAc (3×30 mL). The combined organic layers were washed with saturated NH_4Cl solution (60 mL), dried over Na_2SO_4 , filtered and concentrated *in vacuo*. The crude product was purified by silica gel flash column chromatography using a $\text{CH}_2\text{Cl}_2/\text{MeOH}$ gradient (0% to 20% MeOH) to yield a white solid (129 mg).

Yield 44%; mp: 134-140 $^\circ\text{C}$; ^1H NMR (600 MHz, $\text{DMSO}-d_6$) δ 0.94 (s, 9H, $\text{C}(\text{CH}_3)_3$), 1.24 – 1.27 (m, 4H, CH_2), 1.37 (d, $J = 7.0$ Hz, 3H, CHCH_3), 1.43 – 1.54 (m, 4H, CH_2), 1.77 – 1.83 (m, 1H, 3''-H), 1.92 (quint, $J = 7.5$ Hz, 4H, CH_2 indacene), 1.98 – 2.03 (m, 1H, 3''-H), 2.08 – 2.16 (m, 3H), 2.20 – 2.27 (m, 1H, CH_2 , CH_2CON), 2.45 (s, 3H, CH_3 thiazole), 2.54 (t, $J = 7.4$ Hz, 4H, CH_2 indacene), 2.76 (t, $J = 7.4$ Hz, 4H, CH_2 indacene), 3.58 – 3.64 (m, 2H, 5''-H), 4.27 – 4.30 (m, 1H), 4.33 (d, $J = 6.0$ Hz, 2H), 4.43 (t, $J = 8.0$ Hz, 1H), 4.52 (d, $J = 9.3$ Hz, 1H, 2''H, 4''-H, NHCH , CH_2NHCO), 4.89 – 4.95 (m, 1H, CHCH_3), 5.08 (d, $J = 3.2$ Hz, 1H, OH), 6.91 (s, 1H, $\text{CH}_{\text{arom. indacene}}$), 7.36 – 7.39 (m, 2H), 7.41 – 7.46 (m, 4H), 7.76 (d, $J = 9.2$ Hz, 1H), 7.88 (d, $J = 8.3$ Hz, 2H, $\text{CH}_{\text{arom.}}$, CONH), 8.10 (s, 1H, NHCON), 8.34 – 8.39 (m, 2H, CONH), 8.98 (s, 1H, 2'-H), 10.71 (s, 1H, SO_2NH); ^{13}C NMR (151 MHz, $\text{DMSO}-d_6$) δ 16.0, 22.4, 25.0, 25.1, 25.3, 26.4, 28.4, 28.5, 30.1, 32.4, 34.9, 35.2, 35.3, 37.7, 41.6, 47.7, 56.2, 56.3, 58.5, 68.7, 117.9,

126.4, 127.3, 127.4, 128.7, 128.8, 129.7, 131.1, 137.1, 138.5, 143.0, 144.6, 145.4, 147.7, 149.2, 151.4, 169.6, 170.6, 172.0, 172.3; **LC-MS** (ESI) (90% H₂O to 100% MeCN in 10 min, then 100% MeCN to 15 min, DAD 220-800 nm), t_R = 7.80 min, 96% purity, m/z [M + H]⁺ calcd for C₅₁H₆₅N₇O₈S₂ 968.44, found 968.7; **HRMS** (ESI) m/z [M + H]⁺ calcd for C₅₁H₆₅N₇O₈S₂ 968.4409, found 968.4445.

***N*¹-(4-(*N*-((1,2,3,5,6,7-Hexahydro-*s*-indacen-4-yl)carbamoyl)sulfamoyl)benzyl)-*N*¹⁰-((*S*)-1-((2*S*,4*R*)-4-hydroxy-2-(((*S*)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)carbamoyl)pyrrolidin-1-yl)-3,3-dimethyl-1-oxobutan-2-yl)decanediamide (125)**

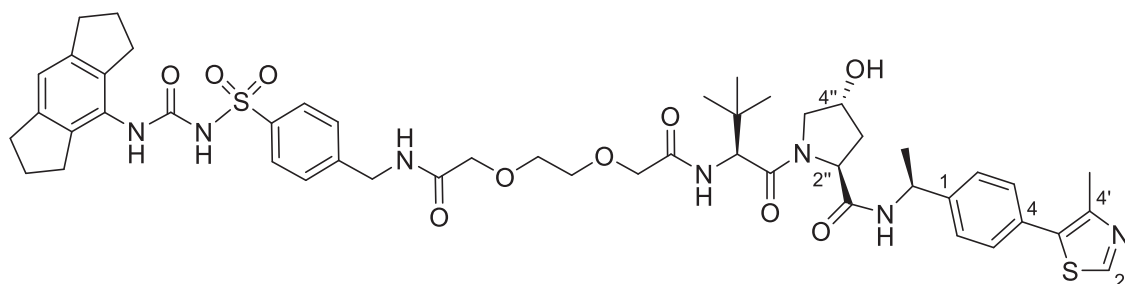


Compound **102** (137 mg, 200 μmol) was dissolved in dry CH₂Cl₂ (3 mL), treated with TFA (3 mL) and stirred at room temperature for 2 h. The mixture was concentrated *in vacuo*. The NLRP3 ligand **5** (97 mg, 200 μmol) was dissolved in dry CH₂Cl₂ (3 mL), treated with TFA (3 mL) and stirred at room temperature for 2 h. The mixture was concentrated *in vacuo*. The deprotected acid compound was dissolved in dry DMF (6 mL). DIPEA (78 mg, 600 μmol) and HATU (84 mg, 220 μmol) were added under argon. The deprotected NLRP3 ligand was dissolved in dry DMF (6 mL) and was added to the mixture, containing the activated acid compound. The reaction mixture was stirred for 2 h at room temperature under argon. The mixture was concentrated *in vacuo*. The crude product was purified by silica gel flash column chromatography using a CH₂Cl₂/MeOH gradient (0% to 10% MeOH) to yield a white solid (88 mg).

Yield 44%; mp: 127-133 °C; ¹H NMR (600 MHz, DMSO-*d*₆) δ 0.94 (s, 9H, C(CH₃)₃), 1.23 – 1.26 (m, 8H, CH₂), 1.37 (d, *J* = 6.9 Hz, 3H, CHCH₃), 1.43 – 1.54 (m, 4H, CH₂), 1.77 – 1.83 (m, 1H, 3''-H), 1.92 (quint, *J* = 7.5 Hz, 4H, CH₂ indacene), 1.98 – 2.03 (m, 1H, 3''-H), 2.08 – 2.16 (m, 3H), 2.21 – 2.27 (m, 1H, CH₂, CH₂CON), 2.45 (s, 3H, CH₃ thiazole), 2.54 (t, *J* = 7.4 Hz, 4H, CH₂ indacene), 2.76 (t, *J* = 7.4 Hz, 4H, CH₂ indacene), 3.57 – 3.64 (m, 2H, 5''-H), 4.26 – 4.30 (m, 1H), 4.33 (d, *J* = 5.9 Hz, 2H), 4.43 (t, *J* = 8.0 Hz, 1H), 4.52 (d, *J* = 9.3 Hz, 1H, 2''-H, 4''),

NHCH₂, CH₂NHCO), 4.89 – 4.95 (m, 1H, CHCH₃), 5.08 (d, $J = 3.6$ Hz, 1H, OH), 6.91 (s, 1H, CH_{arom.} indacene), 7.37 – 7.39 (m, 2H), 7.41 – 7.46 (m, 4H), 7.75 (d, $J = 9.2$ Hz, 1H), 7.85 – 7.89 (m, 2H, CH_{arom.}, CONH), 8.08 (s, 1H, NHCON), 8.33 – 8.40 (m, 2H, CONH), 8.98 (s, 1H, 2'-H), 10.71 (s, 1H, SO₂NH); ¹³C NMR (151 MHz, DMSO-*d*₆) δ 15.9, 22.4, 25.0, 25.2, 25.4, 26.4, 28.6, 28.7, 30.1, 32.4, 34.9, 35.2, 35.3, 37.7, 41.6, 47.7, 56.2, 56.3, 58.5, 68.7, 117.9, 126.4, 127.2, 127.4, 128.7, 128.8, 129.7, 131.1, 137.1, 138.6, 143.0, 144.6, 145.4, 147.7, 149.3, 151.4, 169.6, 170.6, 172.0, 172.3, two signals are missing due to overlapping signals; LC-MS (ESI) (90% H₂O to 100% MeCN in 10 min, then 100% MeCN to 15 min, DAD 200-600 nm), $t_R = 7.10$ min, 95% purity, m/z [M + H]⁺ calcd for C₅₃H₆₉N₇O₈S₂ 996.47, found 996.7; HRMS (ESI) m/z [M + H]⁺ calcd for C₅₃H₆₉N₇O₈S₂ 996.4722, found 996.4738.

(2*S*,4*R*)-1-((*S*)-12-(*tert*-Butyl)-1-(4-(*N*-((1,2,3,5,6,7-hexahydro-*s*-indacen-4-yl)carbamoyl)sulfamoyl)phenyl)-3,10-dioxo-5,8-dioxa-2,11-diazatridecan-13-oyl)-4-hydroxy-*N*-((*S*)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)pyrrolidine-2-carboxamide (126)

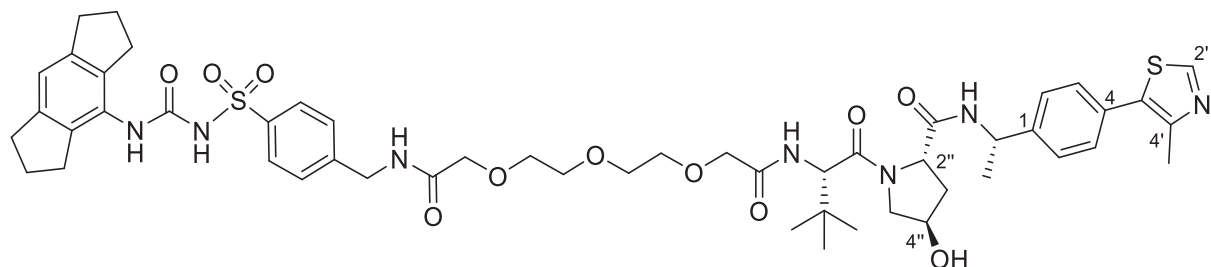


Compound **103** (132 mg, 200 μ mol) was dissolved in dry CH₂Cl₂ (2 mL), treated with TFA (2 mL) and stirred at room temperature for 2 h. The mixture was concentrated *in vacuo*. The NLRP3 ligand **5** (97 mg, 200 μ mol) was dissolved in dry CH₂Cl₂ (2 mL), treated with TFA (2 mL) and stirred at room temperature for 2 h. The mixture was concentrated *in vacuo*. The deprotected acid compound was dissolved in dry DMF (5 mL). DIPEA (78 mg, 600 μ mol) and HATU (84 mg, 220 μ mol) were added under argon. The deprotected NLRP3 ligand was dissolved in dry DMF (5 mL) and was added to the mixture, containing the activated acid compound. The reaction mixture was stirred for 2 h at room temperature under argon. The mixture was concentrated *in vacuo*. The crude product was purified by silica gel flash column chromatography using a CH₂Cl₂/MeOH gradient (0% to 20% MeOH) to yield a white solid (112 mg).

Yield 58%; mp: 123-129 °C; ¹H NMR (500 MHz, DMSO-*d*₆) δ 0.92 (s, 9H, C(CH₃)₃), 1.27 (d, $J = 7.0$ Hz, 3H, CHCH₃), 1.72 – 1.80 (m, 1H, 3''-H), 1.91 (quint, $J = 7.4$ Hz, 4H, CH₂ indacene),

2.04 – 2.12 (m, 1H, 3''-H), 2.45 (s, 3H, CH₃ thiazole), 2.53 (t, $J = 7.4$ Hz, 4H, CH₂ indacene), 2.75 (t, $J = 7.4$ Hz, 4H, CH₂ indacene), 3.55 – 3.63 (m, 2H, 5''-H), 3.63 – 3.70 (m, 4H), 3.93 – 4.04 (m, 4H, OCH₂), 4.25 – 4.32 (m, 1H), 4.36 – 4.45 (m, 2H), 4.48 (t, $J = 8.2$ Hz, 1H), 4.57 (d, $J = 9.6$ Hz, 1H, 2''-H, 4'', NHCH, CH₂NHCO), 4.77 – 4.86 (m, 1H, CHCH₃), 6.91 (s, 1H, CH_{arom.} indacene), 7.30 – 7.36 (m, 2H), 7.38 – 7.47 (m, 3H), 7.47 – 7.53 (m, 2H), 7.86 – 7.90 (m, 2H, CH_{arom.}, CONH), 8.08 (s, 1H, NHCON), 8.39 – 8.47 (m, 2H, CONH), 8.98 (s, 1H, 2'-H), 10.71 (s, 1H, SO₂NH), one signal (1H, OH) is missing; ¹³C NMR (126 MHz, DMSO-*d*₆) δ 15.9, 22.2, 24.9, 26.1, 30.0, 32.4, 35.7, 37.7, 41.4, 47.8, 55.7, 56.5, 58.5, 68.7, 69.5, 70.1, 70.2, 117.9, 126.2, 127.2, 127.7, 128.5, 128.7, 129.6, 131.1, 137.1, 138.4, 143.0, 144.7, 145.2, 147.7, 148.9, 151.3, 168.5, 169.2, 169.3, 170.3, one signal is missing due to overlapping signals; LC-MS (ESI) (90% H₂O to 100% MeCN in 10 min, then 100% MeCN to 15 min, DAD 200-600 nm), $t_R = 5.74$ min, 95% purity, m/z [M + H]⁺ calcd for C₄₉H₆₁N₇O₁₀S₂ 972.40, found 972.7; HRMS (ESI) m/z [M + H]⁺ calcd for C₄₉H₆₁N₇O₁₀S₂ 972.3994, found 972.4015.

(2*S*,4*R*)-1-((*S*)-15-(*tert*-Butyl)-1-(4-(*N*-((1,2,3,5,6,7-hexahydro-*s*-indacen-4-yl)carbamoyl)sulfamoyl)phenyl)-3,13-dioxo-5,8,11-trioxa-2,14-diazahexadecan-16-oyl)-4-hydroxy-*N*-((*S*)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)pyrrolidine-2-carboxamide (127)

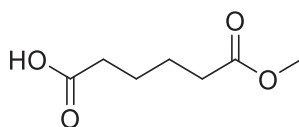


Compound **105** (211 mg, 300 μ mol) was dissolved in dry CH₂Cl₂ (3 mL), treated with TFA (3 mL) and stirred at room temperature for 2 h. The mixture was concentrated *in vacuo*. The NLRP3 ligand **5** (146 mg, 300 μ mol) was dissolved in dry CH₂Cl₂ (3 mL), treated with TFA (3 mL) and stirred at room temperature for 2 h. The mixture was concentrated *in vacuo*. The deprotected acid compound was dissolved in dry DMF (6 mL). DIPEA (116 mg, 900 μ mol) and HATU (125 mg, 330 μ mol) were added under argon. The deprotected NLRP3 ligand was dissolved in dry DMF (6 mL) and was added to the mixture, containing the activated acid compound. The reaction mixture was stirred for 4 h at room temperature under argon. The mixture was concentrated *in vacuo*. The crude product was purified by silica gel flash column

chromatography using a CH₂Cl₂/MeOH gradient (0% to 20% MeOH) to yield a white solid (93 mg).

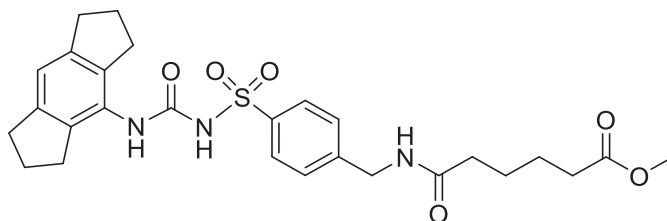
Yield 30%; mp: 103-109 °C; ¹H NMR (600 MHz, DMSO-*d*₆) δ 0.93 (s, 9H, C(CH₃)₃), 1.36 (d, *J* = 7.0 Hz, 3H, CHCH₃), 1.75 – 1.81 (m, 1H, 3''-H), 1.91 (quint, *J* = 7.5 Hz, 4H, CH₂ indacene), 2.01 – 2.08 (m, 1H, 3''-H), 2.45 (s, 3H, CH₃ thiazole), 2.54 (t, *J* = 7.4 Hz, 4H, CH₂ indacene), 2.76 (t, *J* = 7.4 Hz, 4H, CH₂ indacene), 3.55 – 3.65 (m, 10H, 5''-H, OCH₂), 3.93 – 3.99 (m, 4H, OCH₂), 4.26 – 4.30 (m, 1H), 4.39 (d, *J* = 6.1 Hz, 2H), 4.45 (t, *J* = 8.2 Hz, 1H), 4.54 (d, *J* = 9.5 Hz, 1H, 2''-H, 4'', NHCH₂, CH₂NHCO), 4.87 – 4.93 (m, 1H, CHCH₃), 5.09 – 5.14 (m, 1H, OH), 6.90 (s, 1H, CH_{arom.} indacene), 7.33 – 7.40 (m, 3H), 7.41 – 7.45 (m, 2H), 7.47 (d, *J* = 8.2 Hz, 2H), 7.85 – 7.89 (m, 2H, CH_{arom.}, CONH), 8.05 (s, 1H, NHCON), 8.33 – 8.37 (m, 1H), 8.41 (d, *J* = 7.7 Hz, 1H, CONH), 8.97 (s, 1H, 2'-H), one signal (1H, SO₂NH) is missing; ¹³C NMR (151 MHz, DMSO-*d*₆) δ 16.0, 22.4, 25.0, 26.2, 30.1, 32.4, 35.7, 37.7, 41.4, 47.7, 55.7, 56.5, 58.5, 68.7, 69.6, 69.6, 70.0, 70.3, 70.4, 117.8, 126.3, 127.2, 127.5, 128.8, 128.9, 129.7, 131.1, 137.1, 138.9, 143.0, 144.7, 144.9, 147.7, 149.5, 151.4, 168.5, 169.0, 169.5, 170.4, one signal is missing due to overlapping signals; LC-MS (ESI) (90% H₂O to 100% MeCN in 10 min, then 100% MeCN to 20 min, DAD 200-400 nm), *t*_R = 7.41 min, 96% purity, *m/z* [M + H]⁺ calcd for C₅₁H₆₅N₇O₁₁S₂ 1016.43, found 1016.7; HRMS (ESI) *m/z* [M + H]⁺ calcd for C₅₁H₆₅N₇O₁₁S₂ 1016.4256, found 1016.4302.

6-Methoxy-6-oxohexanoic acid (128)



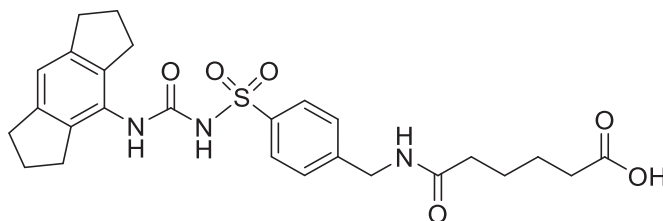
Compound **128** was purchased from Sigma Aldrich/Merck (Darmstadt, Germany).

Methyl

6-((4-(*N*-((1,2,3,5,6,7-hexahydro-*s*-indacen-4-yl)carbamoyl)sulfamoyl)benzyl)amino)-6-oxohexanoate (**129**)

The NLRP3 ligand **5** (486 mg, 1.00 mmol) was dissolved in dry CH_2Cl_2 (15 mL) and was treated with TFA (5 mL). The mixture was stirred for 2 h at room temperature. The mixture was concentrated *in vacuo*. 6-Methoxy-6-oxohexanoic acid (**128**, 160 mg, 1.00 mmol) was dissolved in dry DMF (5 mL). HATU (418 mg, 1.10 mmol) and DIPEA (452 mg, 3.50 mmol) were added under argon. The deprotected NLRP3 ligand was dissolved in dry DMF (5 mL) and was added to the reaction mixture. The reaction mixture was stirred for 18 h at room temperature under argon. The reaction was quenched by the addition of H_2O (25 mL) and was extracted with EtOAc (3×50 mL). The combined organic layers were washed with saturated NH_4Cl solution (75 mL), dried over Na_2SO_4 , filtered and concentrated *in vacuo*. The crude product was purified by silica gel column chromatography using $\text{CH}_2\text{Cl}_2/\text{MeOH}$ (19+1) as eluent to yield an off-white resin (235 mg).

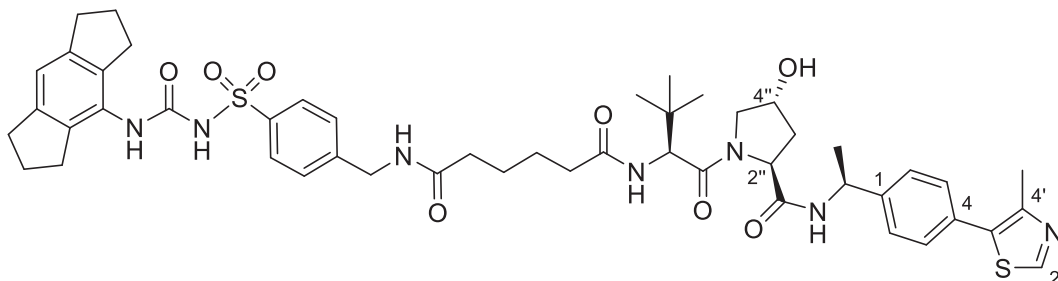
Yield 45%; ^1H NMR (600 MHz, $\text{DMSO}-d_6$) δ 1.50 – 1.55 (m, 4H, CH_2), 1.92 (quint, $J = 7.4$ Hz, 4H, CH_2 indacene), 2.14 – 2.18 (m, 2H), 2.29 – 2.32 (m, 2H, CH_2), 2.53 (t, $J = 7.4$ Hz, 4H, CH_2 indacene), 2.77 (t, $J = 7.4$ Hz, 4H, CH_2 indacene), 3.57 – 3.59 (m, 3H, CH_3), 4.31 – 4.34 (m, 2H, CH_2NH), 6.92 (s, 1H, $\text{CH}_{\text{arom. indacene}}$), 7.45 (d, $J = 8.3$ Hz, 2H), 7.87 (d, $J = 8.3$ Hz, 2H, $\text{CH}_{\text{arom.}}$), 8.07 (s, 1H, NHCON), 8.41 (t, $J = 6.1$ Hz, 1H, CH_2NH), 10.70 (s, 1H, SO_2NH); ^{13}C NMR (151 MHz, $\text{DMSO}-d_6$) δ 24.1, 24.7 (CH_2), 25.0, 30.1, 32.4 (CH_2 indacene), 33.0, 34.9 (CH_2), 41.7 (CH_2NH), 51.2 (CH_3), 118.0, 127.3, 127.5, 128.6, 137.2, 138.5, 143.1, 145.5 ($\text{C}_{\text{arom.}}$), 149.1 (NHCONH), 172.1, 173.3 (CO); LC-MS (ESI) (90% H_2O to 100% MeOH in 10 min, then 100% MeOH to 20 min, DAD 220–400 nm), $t_R = 10.52$ min, 79% purity, m/z $[\text{M} + \text{H}]^+$ calcd for $\text{C}_{27}\text{H}_{33}\text{N}_3\text{O}_6\text{S}$ 528.22, found 528.2.

6-((4-(N-((1,2,3,5,6,7-Hexahydro-s-indacen-4-yl)carbamoyl)sulfamoyl)benzyl)amino)-6-oxohexanoic acid (130)

Compound **129** (211 mg, 400 μ mol) was dissolved in THF (6 mL) and MeOH (2 mL). A solution of lithium hydroxide monohydrate (59 mg, 1.40 mmol) in H₂O (2 mL) was added and the reaction mixture was stirred for 6 h at room temperature. The organic solvents were evaporated *in vacuo* and the aqueous layer was acidified with 2 N HCl to pH~1. The precipitate was collected, washed with H₂O (2 $\times$ 5 mL) and dried under high vacuum to yield a white solid (148 mg).

Yield 72%; mp: decomposition >191 $^{\circ}$ C; **¹H NMR** (500 MHz, DMSO-*d*₆) δ 1.46 – 1.57 (m, 4H, CH₂), 1.87 – 1.98 (m, 4H, CH₂ indacene), 2.13 – 2.17 (m, 2H), 2.18 – 2.23 (m, 2H, CH₂), 2.52 (t, *J* = 7.5 Hz, 4H, CH₂ indacene), 2.76 (t, *J* = 7.4 Hz, 4H, CH₂ indacene), 4.32 (d, *J* = 6.1 Hz, 2H, CH₂NH), 6.91 (s, 1H, CH_{arom.} indacene), 7.44 (d, *J* = 8.1 Hz, 2H), 7.87 (d, *J* = 8.0 Hz, 2H, CH_{arom.}), 8.07 (s, 1H, NHCON), 8.38 (t, *J* = 6.1 Hz, 1H, CH₂NH), 11.97 (s, 1H, COOH), one signal (1H, SO₂NH) is missing due to proton exchange; **¹³C NMR** (126 MHz, DMSO-*d*₆) δ 24.2, 24.8 (CH₂), 25.0, 30.1, 32.4 (CH₂ indacene), 33.4, 35.0 (CH₂), 41.7 (CH₂NH), 118.0, 127.3, 127.4, 128.6, 137.2, 138.4, 143.1, 145.5 (C_{arom.}), 149.0 (NHCONH), 172.2, 174.4 (COO); **LC-MS** (ESI) (90% H₂O + 0.1% AcOH to 100% MeOH + 0.1% AcOH in 10 min, then 100% MeOH + 0.1% AcOH to 20 min, DAD 220-400 nm), *t*_R = 10.75 min, 96% purity, *m/z* [M + H]⁺ calcd for C₂₆H₃₁N₃O₆S 514.20, found 514.5.

***N*¹-(4-(*N*-((1,2,3,5,6,7-Hexahydro-*s*-indacen-4-yl)carbamoyl)sulfamoyl)benzyl)-*N*⁶-((*S*)-1-((2*S*,4*R*)-4-hydroxy-2-(((*S*)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)carbamoyl)pyrrolidin-1-yl)-3,3-dimethyl-1-oxobutan-2-yl)adipamide (131)**

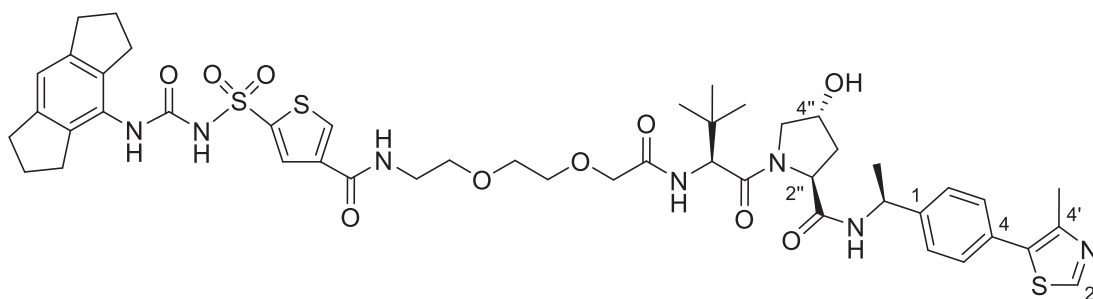


The VHL ligand **26** (136 mg, 250 μ mol) was dissolved in dry CH_2Cl_2 (5 mL), treated with TFA (5 mL) and stirred at room temperature for 2 h. The mixture was concentrated *in vacuo*. Compound **130** (128 mg, 250 μ mol) was dissolved in dry DMF (5 mL). DIPEA (114 mg, 880 μ mol) and HATU (106 mg, 280 μ mol) were added under argon. The deprotected VHL ligand **96** was dissolved in dry DMF (5 mL) and was added to the mixture, containing the activated acid compound. The reaction mixture was stirred for 18 h at room temperature under argon. The mixture was concentrated *in vacuo*. The residue was diluted with H_2O (25 mL) and was extracted with EtOAc (3×50 mL). The combined organic layers were washed with brine (75 mL), dried over Na_2SO_4 , filtered and concentrated *in vacuo*. The crude product was purified by silica gel flash column chromatography using a $\text{CH}_2\text{Cl}_2/\text{MeOH}$ gradient (0% to 20% MeOH) to yield a white solid (100 mg).

Yield 43%; mp: 136-142 $^\circ\text{C}$; ^1H NMR (600 MHz, $\text{DMSO}-d_6$) δ 0.93 (s, 9H, $\text{C}(\text{CH}_3)_3$), 1.37 (d, $J = 6.9$ Hz, 3H, CHCH_3), 1.45 – 1.54 (m, 4H, CH_2), 1.76 – 1.83 (m, 1H, 3''-H), 1.92 (quint, $J = 7.5$ Hz, 4H, CH_2 indacene), 1.98 – 2.03 (m, 1H, 3''-H), 2.10 – 2.17 (m, 3H), 2.23 – 2.29 (m, 1H, CH_2 , CH_2CON), 2.45 (s, 3H, CH_3 thiazole), 2.54 (t, $J = 7.4$ Hz, 4H, CH_2 indacene), 2.76 (t, $J = 7.3$ Hz, 4H, CH_2 indacene), 3.56 – 3.65 (m, 2H, 5''-H), 4.26 – 4.30 (m, 1H), 4.33 (d, $J = 6.0$ Hz, 2H), 4.43 (t, $J = 8.0$ Hz, 1H), 4.52 (d, $J = 9.3$ Hz, 1H, 2''-H, 4''-H, NHCH , CH_2NHCO), 4.88 – 4.95 (m, 1H, CHCH_3), 5.08 (d, $J = 3.4$ Hz, 1H, OH), 6.91 (s, 1H, $\text{CH}_{\text{arom. indacene}}$), 7.36 – 7.40 (m, 2H), 7.41 – 7.46 (m, 4H), 7.77 (d, $J = 9.2$ Hz, 1H), 7.86 (d, $J = 8.2$ Hz, 2H, $\text{CH}_{\text{arom.}}$, CONH), 8.02 (s, 1H, NHCON), 8.31 – 8.41 (m, 2H, CONH), 8.98 (s, 1H, 2'-H), 10.71 (s, 1H, SO_2NH); ^{13}C NMR (151 MHz, $\text{DMSO}-d_6$) δ 15.9, 22.4, 25.0, 25.0, 25.2, 26.4, 30.1, 32.4, 34.7, 35.1, 35.2, 37.7, 41.6, 47.7, 56.2, 56.4, 58.5, 68.7, 117.8, 126.3, 127.2, 127.3, 128.8, 129.7, 131.1, 137.1, 143.0, 144.6, 147.7, 151.4, 169.6, 170.6, 171.8, 172.2; LC-MS (ESI) (90% H_2O to 100% MeCN in 10 min, then 100% MeCN to 15 min, DAD 220-800 nm), $t_R = 5.87$ min, 92% purity,

m/z $[M + H]^+$ calcd for $C_{49}H_{61}N_7O_8S_2$ 940.41, found 940.9; **HRMS** (ESI) m/z $[M + H]^+$ calcd for $C_{49}H_{61}N_7O_8S_2$ 940.4096, found 940.4081.

(2*S*,4*R*)-1-((*S*)-12-(*tert*-Butyl)-1-(5-(*N*-((1,2,3,5,6,7-hexahydro-*s*-indacen-4-yl)carbamoyl)sulfamoyl)thiophen-3-yl)-1,10-dioxo-5,8-dioxo-2,11-diazatridecan-13-oyl)-4-hydroxy-*N*-((*S*)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)pyrrolidine-2-carboxamide (132)

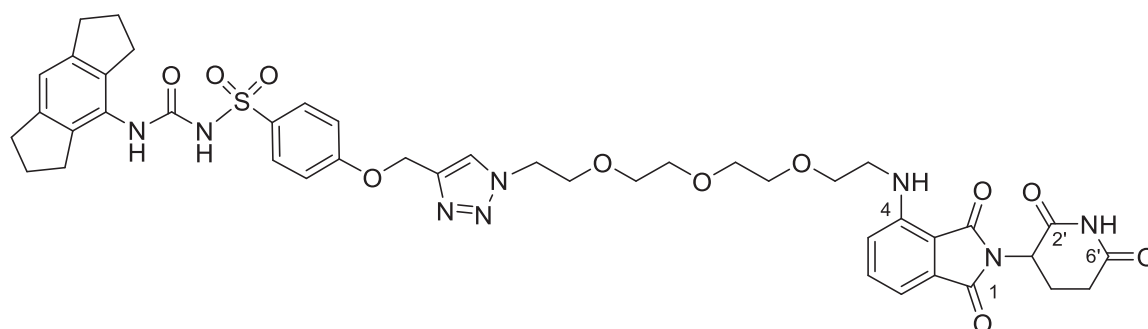


Compound **107** (138 mg, 200 μ mol) was dissolved in dry CH_2Cl_2 (2 mL), treated with TFA (2 mL) and stirred at room temperature for 2 h. The mixture was concentrated *in vacuo*. The NLRP3 ligand **8** (81 mg, 200 μ mol) was dissolved in dry DMF (3 mL). DIPEA (78 mg, 600 μ mol) and HATU (84 mg, 220 μ mol) were added under argon. The deprotected VHL precursor was dissolved in dry DMF (3 mL) and was added to the mixture, containing the activated acid compound. The reaction mixture was stirred for 4 h at room temperature under argon. The mixture was concentrated *in vacuo*. The crude product was purified by silica gel flash column chromatography using a CH_2Cl_2 /MeOH gradient (0% to 20% MeOH) to yield a white solid (110 mg).

Yield 56%; mp: 103-109 $^{\circ}C$; ^1H NMR (600 MHz, $DMSO-d_6$) δ 0.93 (s, 9H, $C(CH_3)_3$), 1.34 (d, $J = 7.0$ Hz, 3H, $CHCH_3$), 1.75 – 1.81 (m, 1H, 3''-H), 1.92 (quint, $J = 7.4$ Hz, 4H, CH_2 indacene), 2.02 – 2.09 (m, 1H, 3''-H), 2.45 (s, 3H, CH_3 thiazole), 2.59 (t, $J = 7.4$ Hz, 4H, CH_2 indacene), 2.77 (t, $J = 7.4$ Hz, 4H, CH_2 indacene), 3.39 – 3.43 (m, 2H), 3.53 – 3.65 (m, 8H, 5''-H, CH_2), 3.96 (s, 2H, OCH_2), 4.26 – 4.31 (m, 1H), 4.46 (t, $J = 8.2$ Hz, 1H), 4.55 (d, $J = 9.5$ Hz, 1H, 2''-H, 4''-H, $NHCH$), 4.86 – 4.92 (m, 1H, $CHCH_3$), 5.12 (s, 1H, OH), 6.93 (s, 1H, $CH_{arom.}$ indacene), 7.32 – 7.46 (m, 5H, $CH_{arom.}$, CONH), 8.15 (d, $J = 1.6$ Hz, 1H, $CH_{arom.}$ thiophene), 8.19 (s, 1H, $NHCON$), 8.40 (d, $J = 7.7$ Hz, 1H, CONH), 8.48 (d, $J = 1.7$ Hz, 1H, $CH_{arom.}$ thiophene), 8.62 (t, $J = 5.7$ Hz, 1H, CONH), 8.97 (s, 1H, 2'-H), 11.03 (s, 1H, SO_2NH); ^{13}C NMR (151 MHz, $DMSO-d_6$) δ 16.0, 22.3, 25.0, 26.2, 30.1, 32.4, 35.7, 37.7, 41.8, 47.7, 53.6, 55.7, 56.5, 58.6, 68.7, 69.0, 69.3, 69.6, 70.4, 118.0, 126.3, 128.8, 129.7, 131.1, 132.1, 135.5, 136.7, 137.3, 143.1, 144.7, 147.7,

149.2, 151.4, 160.9, 168.5, 169.1, 170.4; **LC-MS** (ESI) (90% H₂O to 100% MeCN in 10 min, then 100% MeCN to 15 min, DAD 220-800 nm), t_R = 5.77 min, 96% purity, m/z [M + H]⁺ calcd for C₄₇H₅₉N₇O₁₀S₃ 978.36, found 987.8; **HRMS** (ESI) m/z [M + H]⁺ calcd for C₄₇H₅₉N₇O₁₀S₃ 978.3558, found 978.3566.

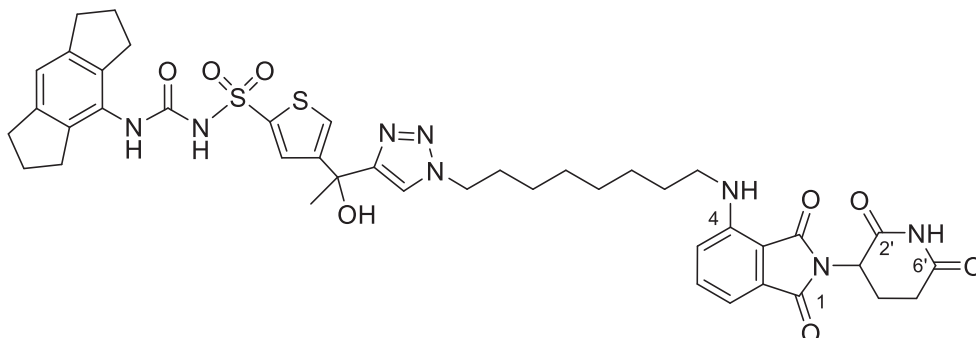
4-((1-(2-(2-(2-(2-((2-(2,6-Dioxopiperidin-3-yl)-1,3-dioxoisindolin-4-yl)amino)ethoxy)ethoxy)ethoxy)ethyl)-1*H*-1,2,3-triazol-4-yl)methoxy)-*N*-((1,2,3,5,6,7-hexahydro-*s*-indacen-4-yl)carbamoyl)benzenesulfonamide (133)



Compound **133** was synthesized following the general procedure for CuAAC but using NLRP3 ligand **12** (200 μ mol) and azido-precursor **92** (200 μ mol). The crude product was purified by silica gel flash column chromatography using a CH₂Cl₂/MeOH gradient (0% to 20% MeOH) to yield a yellow solid (115 mg).

Yield 65%; mp: 92-98 °C; ¹H NMR (600 MHz, DMSO-*d*₆) δ 1.91 (quint, J = 7.4 Hz, 4H, CH₂ indacene), 1.99 – 2.05 (m, 1H, 4'-H), 2.51 – 2.61 (m, 6H, 4'-H, 5'-H, CH₂ indacene), 2.76 (t, J = 7.4 Hz, 4H, CH₂ indacene), 2.84 – 2.91 (m, 1H, 5'-H), 3.41 – 3.45 (m, 2H), 3.46 – 3.51 (m, 6H), 3.52 – 3.54 (m, 2H), 3.58 (t, J = 5.4 Hz, 2H), 3.80 (t, J = 5.2 Hz, 2H), 4.52 (t, J = 5.2 Hz, 2H, OCH₂, CH₂NH, CH₂N), 5.04 (dd, J = 12.8, 5.4 Hz, 1H, 3'-H), 5.23 (s, 2H, OCH₂CNN), 6.58 (t, J = 5.9 Hz, 1H, NHCH₂), 6.91 (s, 1H, CH_{arom.} indacene), 7.03 (d, J = 7.0 Hz, 1H), 7.11 (d, J = 8.6 Hz, 1H, 5-H, 7-H), 7.21 – 7.24 (m, 2H, CH_{arom.}), 7.56 (dd, J = 8.3, 7.3 Hz, 1H, 6-H), 7.85 – 7.89 (m, 2H, CH_{arom.}), 8.03 (s, 1H, NHCON), 8.21 (s, 1H, CH_{arom.} triazole), 10.60 (s, 1H, SO₂NH), 11.08 (s, 1H, NH imide); ¹³C NMR (151 MHz, DMSO-*d*₆) δ 22.1, 25.0, 30.1, 31.0, 32.4, 41.7, 48.5, 49.4, 61.5, 68.6, 68.8, 69.5, 69.7, 69.7, 69.7, 109.2, 110.6, 114.7, 117.4, 117.9, 125.1, 128.7, 129.5, 132.1, 136.2, 137.1, 141.8, 143.0, 146.4, 149.2, 161.5, 167.2, 168.9, 169.5, 170.0, 172.8; **LC-MS** (ESI) (90% H₂O to 100% MeCN in 10 min, then 100% MeCN to 15 min, DAD 200-600 nm), t_R = 5.98 min, 96% purity, m/z [M + H]⁺ calcd for C₄₃H₄₈N₈O₁₁S 885.32, found 885.6; **HRMS** (ESI) m/z [M + H]⁺ calcd for C₄₃H₄₈N₈O₁₁S 885.3236, found 885.3275.

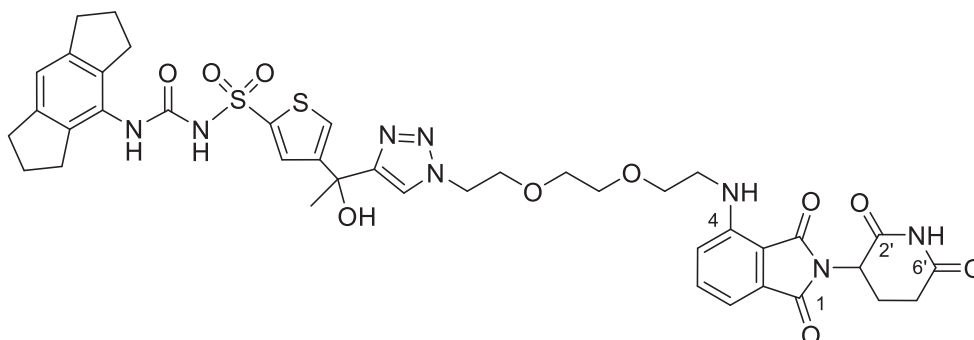
4-(1-(1-(8-((2-(2,6-Dioxopiperidin-3-yl)-1,3-dioxoisindolin-4-yl)amino)octyl)-1*H*-1,2,3-triazol-4-yl)-1-hydroxyethyl)-*N*-((1,2,3,5,6,7-hexahydro-*s*-indacen-4-yl)carbamoyl)thiophene-2-sulfonamide (135)



Compound **135** was synthesized following the general procedure for CuAAC and using azido-precursor **90** (150 μ mol). The crude product was purified by silica gel column chromatography using a $\text{CH}_2\text{Cl}_2/\text{MeOH}$ gradient (0% to 20% MeOH) to yield a yellow solid (65 mg).

Yield 50%; mp: 137-143 $^{\circ}\text{C}$; $^1\text{H NMR}$ (600 MHz, $\text{DMSO}-d_6$) δ 1.21 – 1.34 (m, 8H), 1.51 – 1.59 (m, 2H, CH_2), 1.73 – 1.81 (m, 5H, CH_3 , CH_2), 1.92 (quint, $J = 7.4$ Hz, 4H, CH_2 indacene), 2.00 – 2.05 (m, 1H, 4'-H), 2.51 – 2.62 (m, 6H, 4'-H, 5'-H, CH_2 indacene), 2.78 (t, $J = 7.4$ Hz, 4H, CH_2 indacene), 2.84 – 2.92 (m, 1H, 5'-H), 3.25 – 3.29 (m, 2H), 4.26 (t, $J = 7.2$ Hz, 2H, CH_2), 5.04 (dd, $J = 12.8, 5.4$ Hz, 1H, 3'-H), 6.05 (s, 1H, OH), 6.50 (t, $J = 5.9$ Hz, 1H, CH_2NH), 6.92 (s, 1H, $\text{CH}_{\text{arom. indacene}}$), 7.01 (d, $J = 7.0$ Hz, 1H), 7.08 (d, $J = 8.6$ Hz, 1H, 5-H, 7-H), 7.55 – 7.60 (m, 1H, 6-H), 7.68 (s, 1H), 7.71 (s, 1H, $\text{CH}_{\text{arom. thiophene}}$), 7.87 (s, 1H, $\text{CH}_{\text{arom. triazole}}$), 8.07 (s, 1H, NHCO), 10.85 (s, 1H, SO_2NH), 11.07 (s, 1H, NH_{imide}); $^{13}\text{C NMR}$ (151 MHz, $\text{DMSO}-d_6$) δ 22.1, 25.0, 25.8, 26.2, 28.3, 28.5, 28.6, 29.6, 30.1, 30.6, 31.0, 32.4, 41.8, 48.5, 49.2, 69.5, 109.0, 110.3, 117.1, 117.8, 121.1, 127.1, 128.9, 131.7, 132.2, 136.2, 137.2, 140.8, 143.0, 146.4, 149.8, 153.9, 167.3, 168.9, 170.0, 172.7; **LC-MS** (ESI) (90% H_2O to 100% MeCN in 10 min, then 100% MeCN to 15 min, DAD 200-600 nm), $t_R = 6.06$ min, 96% purity, m/z $[\text{M} + \text{H}]^+$ calcd for $\text{C}_{42}\text{H}_{48}\text{N}_8\text{O}_8\text{S}_2$ 857.31, found 857.5; **HRMS** (ESI) m/z $[\text{M} + \text{H}]^+$ calcd for $\text{C}_{42}\text{H}_{48}\text{N}_8\text{O}_8\text{S}_2$ 857.3109, found 857.3104.

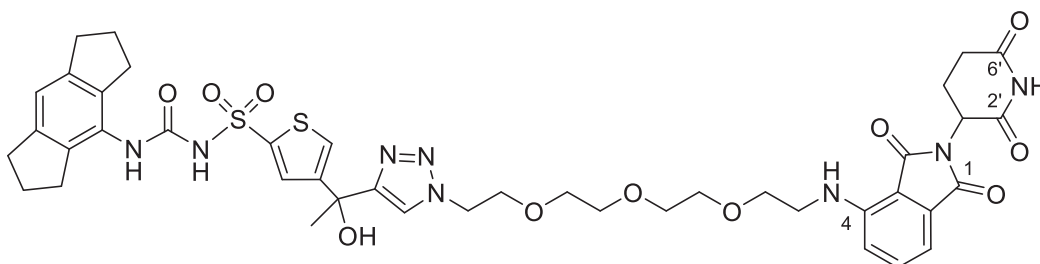
4-(1-(1-(2-(2-(2-((2-(2,6-Dioxopiperidin-3-yl)-1,3-dioxoisindolin-4-yl)amino)ethoxy)ethoxy)ethyl)-1*H*-1,2,3-triazol-4-yl)-1-hydroxyethyl)-*N*-((1,2,3,5,6,7-hexahydro-*s*-indacen-4-yl)carbamoyl)thiophene-2-sulfonamide (136)



Compound **136** was synthesized following the general procedure for CuAAC and using azido-precursor **91** (200 μ mol). The crude product was purified by silica gel column chromatography using a $\text{CH}_2\text{Cl}_2/\text{MeOH}$ gradient (0% to 20% MeOH) to yield a yellow solid (106 mg).

Yield 62%; mp: 142-148 $^\circ\text{C}$; $^1\text{H NMR}$ (600 MHz, $\text{DMSO}-d_6$) δ 1.78 (s, 3H, CH_3), 1.88 – 1.96 (m, 4H, CH_2 indacene), 1.99 – 2.04 (m, 1H, 4'-H), 2.51 – 2.64 (m, 6H, 4'-H, 5'-H, CH_2 indacene), 2.77 (t, $J = 7.2$ Hz, 4H, CH_2 indacene), 2.83 – 2.91 (m, 1H, 5'-H), 3.41 – 3.45 (m, 2H), 3.49 – 3.53 (m, 4H), 3.56 (t, $J = 5.4$ Hz, 2H), 3.79 (t, $J = 5.1$ Hz, 2H), 4.45 (t, $J = 5.3$ Hz, 2H, CH_2), 5.04 (dd, $J = 12.8, 5.4$ Hz, 1H, 3'-H), 6.10 (s, 1H, OH), 6.58 (t, $J = 5.9$ Hz, 1H, CH_2NH), 6.91 (s, 1H, $\text{CH}_{\text{arom. indacene}}$), 7.03 (d, $J = 7.0$ Hz, 1H), 7.12 (d, $J = 8.5$ Hz, 1H, 5-H, 7-H), 7.54 – 7.59 (m, 1H, 6-H), 7.60 – 7.80 (m, 2H, $\text{CH}_{\text{arom. thiophene}}$), 7.88 (s, 1H, $\text{CH}_{\text{arom. triazole}}$), 10.89 (s, 1H, SO_2NH), 11.07 (s, 1H, NH_{imide}), one signal (1H, NHCO) is missing; $^{13}\text{C NMR}$ (151 MHz, $\text{DMSO}-d_6$) δ 22.1, 25.0, 30.1, 30.5, 30.9, 32.4, 41.6, 48.5, 49.3, 68.6, 68.8, 69.5, 69.5, 109.2, 110.6, 117.4, 117.7, 121.7, 127.2, 132.0, 136.1, 137.1, 142.9, 146.3, 149.9, 154.0, 167.2, 168.9, 170.0, 172.7; **LC-MS** (ESI) (90% H_2O to 100% MeCN in 10 min, then 100% MeCN to 15 min, DAD 200-600 nm), $t_R = 4.96$ min, 96% purity, m/z $[\text{M} + \text{H}]^+$ calcd for $\text{C}_{40}\text{H}_{44}\text{N}_8\text{O}_{10}\text{S}_2$ 861.27, found 861.5; **HRMS** (ESI) m/z $[\text{M} + \text{H}]^+$ calcd for $\text{C}_{40}\text{H}_{44}\text{N}_8\text{O}_{10}\text{S}_2$ 861.2695, found 861.2701.

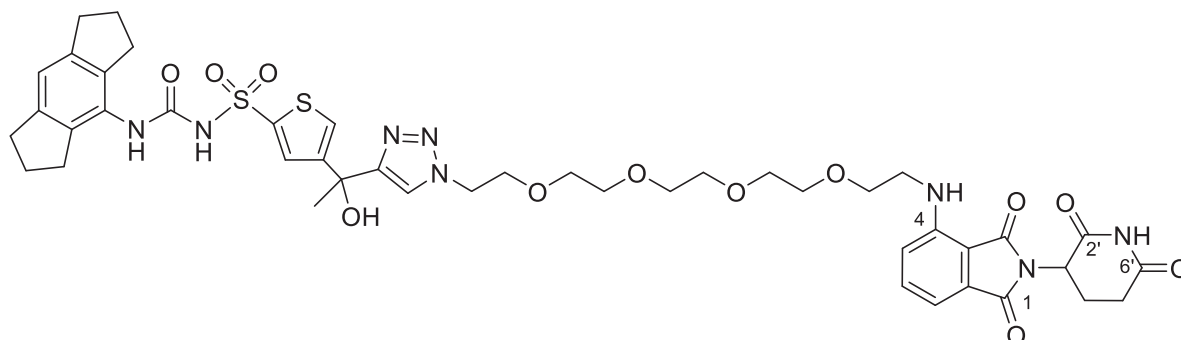
4-(1-(1-(2-(2-(2-(2-((2-(2,6-Dioxopiperidin-3-yl)-1,3-dioxoisindolin-4-yl)amino)ethoxy)ethoxy)ethoxy)ethyl)-1*H*-1,2,3-triazol-4-yl)-1-hydroxyethyl)-*N*-((1,2,3,5,6,7-hexahydro-*s*-indacen-4-yl)carbamoyl)thiophene-2-sulfonamide (137)



Compound **137** was synthesized following the general procedure for CuAAC and using azido-precursor **92** (200 μ mol). The crude product was purified by silica gel column chromatography using a $\text{CH}_2\text{Cl}_2/\text{MeOH}$ gradient (0% to 20% MeOH) to yield a yellow solid (80 mg).

Yield 44%; mp: 145-151 $^\circ\text{C}$; $^1\text{H NMR}$ (600 MHz, $\text{DMSO}-d_6$) δ 1.79 (s, 3H, CH_3), 1.93 (quint, $J = 7.4$ Hz, 4H, CH_2 indacene), 1.99 – 2.05 (m, 1H, 4'-H), 2.52 – 2.62 (m, 6H, 4'-H, 5'-H, CH_2 indacene), 2.78 (t, $J = 7.4$ Hz, 4H, CH_2 indacene), 2.83 – 2.91 (m, 1H, 5'-H), 3.42 – 3.50 (m, 8H), 3.51 – 3.54 (m, 2H), 3.60 (t, $J = 5.4$ Hz, 2H), 3.77 (t, $J = 5.3$ Hz, 2H), 4.44 (t, $J = 5.0$ Hz, 2H, CH_2), 5.04 (dd, $J = 12.9, 5.4$ Hz, 1H, 3'-H), 6.10 (s, 1H, OH), 6.58 (t, $J = 5.8$ Hz, 1H, CH_2NH), 6.93 (s, 1H, $\text{CH}_{\text{arom. indacene}}$), 7.03 (d, $J = 7.0$ Hz, 1H), 7.12 (d, $J = 8.6$ Hz, 1H, 5-H, 7-H), 7.54 – 7.59 (m, 1H, 6-H), 7.71 (s, 1H), 7.74 (s, 1H, $\text{CH}_{\text{arom. thiophene}}$), 7.86 (s, 1H, $\text{CH}_{\text{arom. triazole}}$), 8.10 (s, 1H, NHCO), 10.89 (s, 1H, SO_2NH), 11.08 (s, 1H, NH imide); $^{13}\text{C NMR}$ (151 MHz, $\text{DMSO}-d_6$) δ 22.1, 25.0, 30.1, 30.6, 31.0, 32.4, 41.7, 48.6, 49.3, 68.6, 68.9, 69.5, 69.7, 69.7, 109.2, 110.7, 117.4, 118.0, 121.6, 127.6, 128.6, 132.1, 132.1, 136.2, 137.3, 140.1, 143.1, 146.4, 149.0, 149.9, 153.9, 167.3, 168.9, 170.1, 172.8; **LC-MS** (ESI) (90% H_2O to 100% MeCN in 10 min, then 100% MeCN to 15 min, DAD 200-600 nm), $t_R = 5.44$ min, 98% purity, m/z $[\text{M} + \text{H}]^+$ calcd for $\text{C}_{42}\text{H}_{48}\text{N}_8\text{O}_{11}\text{S}_2$ 905.30, found 905.6; **HRMS** (ESI) m/z $[\text{M} + \text{H}]^+$ calcd for $\text{C}_{42}\text{H}_{48}\text{N}_8\text{O}_{11}\text{S}_2$ 905.2957, found 905.2976.

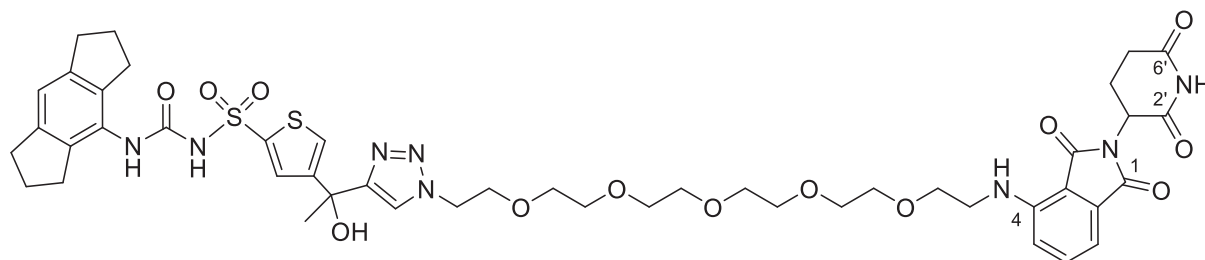
4-(1-(1-(14-((2-(2,6-Dioxopiperidin-3-yl)-1,3-dioxoisindolin-4-yl)amino)-3,6,9,12-tetraoxatetradecyl)-1*H*-1,2,3-triazol-4-yl)-1-hydroxyethyl)-*N*-((1,2,3,5,6,7-hexahydro-*s*-indacen-4-yl)carbamoyl)thiophene-2-sulfonamide (138)



Compound **138** was synthesized following the general procedure for CuAAC and using azido-precursor **93** (200 μ mol). The crude product was purified by silica gel column chromatography using a $\text{CH}_2\text{Cl}_2/\text{MeOH}$ gradient (0% to 20% MeOH) to yield a yellow solid (105 mg).

Yield 55%; mp: 104-110 $^\circ\text{C}$; $^1\text{H NMR}$ (600 MHz, $\text{DMSO}-d_6$) δ 1.79 (s, 3H, CH_3), 1.93 (quint, $J = 7.4$ Hz, 4H, CH_2 indacene), 1.99 – 2.05 (m, 1H, 4'-H), 2.51 – 2.62 (m, 6H, 4'-H, 5'-H, CH_2 indacene), 2.78 (t, $J = 7.4$ Hz, 4H, CH_2 indacene), 2.84 – 2.92 (m, 1H, 5'-H), 3.43 – 3.49 (m, 10H), 3.50 – 3.52 (m, 2H), 3.53 – 3.56 (m, 2H), 3.61 (t, $J = 5.5$ Hz, 2H), 3.77 (t, $J = 5.3$ Hz, 2H), 4.43 – 4.47 (m, 2H, CH_2), 5.05 (dd, $J = 12.8, 5.4$ Hz, 1H, 3'-H), 6.10 (s, 1H, OH), 6.59 (t, $J = 5.9$ Hz, 1H, CH_2NH), 6.94 (s, 1H, $\text{CH}_{\text{arom. indacene}}$), 7.04 (d, $J = 7.0$ Hz, 1H), 7.13 (d, $J = 8.6$ Hz, 1H, 5-H, 7-H), 7.57 (dd, $J = 8.4, 7.2$ Hz, 1H, 6-H), 7.72 (d, $J = 1.7$ Hz, 1H), 7.75 (d, $J = 1.7$ Hz, 1H, $\text{CH}_{\text{arom. thiophene}}$), 7.87 (s, 1H, $\text{CH}_{\text{arom. triazole}}$), 8.11 (s, 1H, CONH), 10.85 (s, 1H, SO_2NH), 11.07 (s, 1H, NH imide); $^{13}\text{C NMR}$ (151 MHz, $\text{DMSO}-d_6$) δ 22.1, 25.0, 30.1, 30.6, 31.0, 32.4, 41.7, 48.5, 49.3, 68.6, 68.9, 69.5, 69.6, 69.7, 69.7, 69.8, 109.2, 110.6, 117.4, 118.0, 121.6, 127.6, 128.6, 132.1, 132.1, 136.2, 137.2, 140.0, 143.1, 146.4, 148.9, 149.9, 153.9, 167.3, 168.9, 170.0, 172.7; **LC-MS** (ESI) (90% H_2O to 100% MeCN in 10 min, then 100% MeCN to 15 min, DAD 200-600 nm), $t_R = 5.13$ min, 96% purity, m/z $[\text{M} + \text{H}]^+$ calcd for $\text{C}_{44}\text{H}_{52}\text{N}_8\text{O}_{12}\text{S}_2$ 949.32, found 949.6; **HRMS** (ESI) m/z $[\text{M} + \text{H}]^+$ calcd for $\text{C}_{44}\text{H}_{52}\text{N}_8\text{O}_{12}\text{S}_2$ 949.3219, found 949.3212.

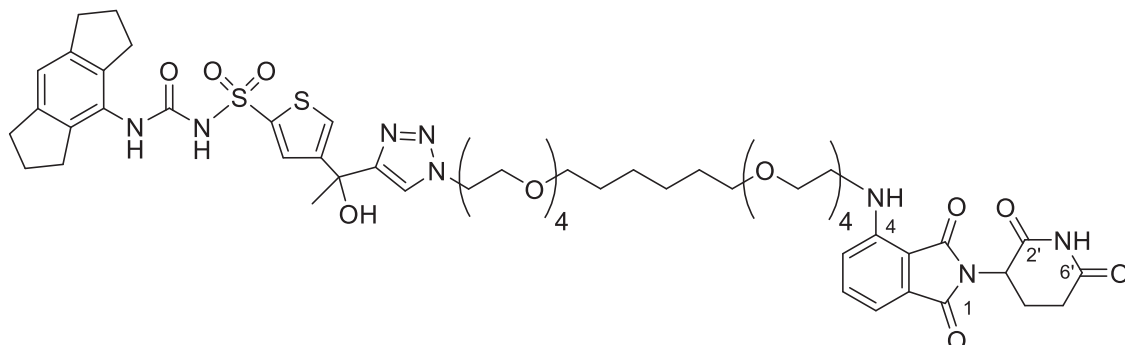
4-(1-(1-(17-((2-(2,6-Dioxopiperidin-3-yl)-1,3-dioxoisindolin-4-yl)amino)-3,6,9,12,15-pentaoxaheptadecyl)-1*H*-1,2,3-triazol-4-yl)-1-hydroxyethyl)-*N*-((1,2,3,5,6,7-hexahydro-*s*-indacen-4-yl)carbamoyl)thiophene-2-sulfonamide (139)



Compound **139** was synthesized following the general procedure for CuAAC and using azido-precursor **94** (200 μ mol). The crude product was purified by silica gel column chromatography using a $\text{CH}_2\text{Cl}_2/\text{MeOH}$ gradient (0% to 20% MeOH) to yield a yellow solid (82 mg).

Yield 44%; mp: 100-106 $^{\circ}\text{C}$; ^1H NMR (600 MHz, $\text{DMSO}-d_6$) δ 1.79 (s, 3H, CH_3), 1.93 (quint, $J = 7.4$ Hz, 4H, CH_2 indacene), 2.00 – 2.05 (m, 1H, 4'-H), 2.51 – 2.62 (m, 6H, 4'-H, 5'-H, CH_2 indacene), 2.78 (t, $J = 7.4$ Hz, 4H, CH_2 indacene), 2.84 – 2.92 (m, 1H, 5'-H), 3.43 – 3.50 (m, 14H), 3.51 – 3.53 (m, 2H), 3.54 – 3.57 (m, 2H), 3.61 (t, $J = 5.5$ Hz, 2H), 3.78 (t, $J = 5.3$ Hz, 2H), 4.46 (t, $J = 5.3$ Hz, 2H, CH_2), 5.05 (dd, $J = 12.8, 5.4$ Hz, 1H, 3'-H), 6.09 (s, 1H, OH), 6.59 (t, $J = 5.9$ Hz, 1H, CH_2NH), 6.94 (s, 1H, $\text{CH}_{\text{arom. indacene}}$), 7.04 (d, $J = 7.0$ Hz, 1H), 7.14 (d, $J = 8.6$ Hz, 1H, 5-H, 7-H), 7.57 (dd, $J = 8.3, 7.3$ Hz, 1H, 6-H), 7.72 (d, $J = 1.6$ Hz, 1H), 7.75 (d, $J = 1.5$ Hz, 1H, $\text{CH}_{\text{arom. thiophene}}$), 7.87 (s, 1H, $\text{CH}_{\text{arom. triazole}}$), 8.10 (s, 1H, CONH), 10.87 (s, 1H, SO_2NH), 11.07 (s, 1H, NH imide); ^{13}C NMR (151 MHz, $\text{DMSO}-d_6$) δ 22.1, 25.0, 30.1, 30.6, 31.0, 32.4, 41.7, 48.5, 49.3, 68.6, 68.9, 69.5, 69.6, 69.7, 69.7, 69.8, 109.2, 110.6, 117.4, 118.0, 121.6, 127.6, 128.6, 132.0, 132.1, 136.2, 137.2, 140.1, 143.1, 146.4, 149.0, 149.9, 153.9, 167.3, 168.9, 170.0, 172.7; LC-MS (ESI) (90% H_2O to 100% MeCN in 10 min, then 100% MeCN to 15 min, DAD 200-600 nm), $t_R = 5.74$ min, 95% purity, m/z $[\text{M} + \text{H}]^+$ calcd for $\text{C}_{46}\text{H}_{56}\text{N}_8\text{O}_{13}\text{S}_2$ 993.35, found 993.6; HRMS (ESI) m/z $[\text{M} + \text{H}]^+$ calcd for $\text{C}_{46}\text{H}_{56}\text{N}_8\text{O}_{13}\text{S}_2$ 993.3481, found 993.3468.

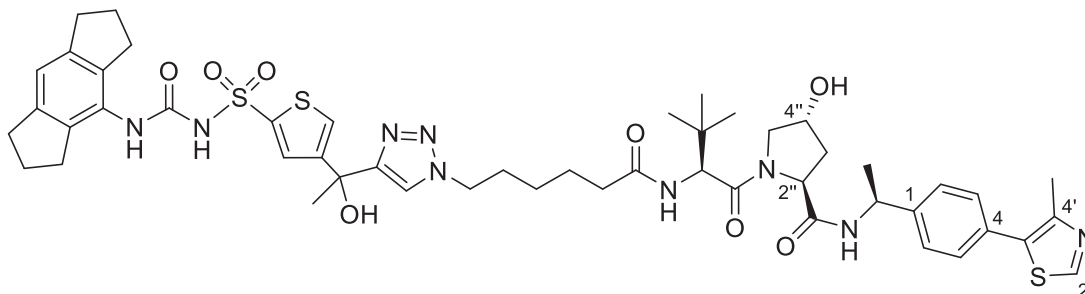
4-(1-(1-(30-((2-(2,6-Dioxopiperidin-3-yl)-1,3-dioxoisindolin-4-yl)amino)-3,6,9,12,19,22,25,28-octaaxatriacontyl)-1*H*-1,2,3-triazol-4-yl)-1-hydroxyethyl)-*N*-((1,2,3,5,6,7-hexahydro-*s*-indacen-4-yl)carbamoyl)thiophene-2-sulfonamide (140)



Compound **140** was synthesized following the general procedure for CuAAC and using azido-precursor **95** (200 μ mol). The crude product was purified by silica gel column chromatography using a $\text{CH}_2\text{Cl}_2/\text{MeOH}$ gradient (0% to 20% MeOH) to yield a yellow solid (111 mg).

Yield 47%; mp: 88-94 $^\circ\text{C}$; ^1H NMR (600 MHz, $\text{DMSO}-d_6$) δ 1.24 – 1.27 (m, 4H), 1.41 – 1.49 (m, 4H, CH_2), 1.79 (s, 3H, CH_3), 1.93 (quint, $J = 7.4$ Hz, 4H, CH_2 indacene), 1.99 – 2.05 (m, 1H, 4'-H), 2.51 – 2.62 (m, 6H, 4'-H, 5'-H, CH_2 indacene), 2.78 (t, $J = 7.4$ Hz, 4H, CH_2 indacene), 2.84 – 2.92 (m, 1H, 5'-H), 3.32 – 3.35 (m, 4H), 3.41 – 3.52 (m, 22H), 3.52 – 3.54 (m, 2H), 3.55 – 3.58 (m, 2H), 3.62 (t, $J = 5.5$ Hz, 2H), 3.78 (t, $J = 5.3$ Hz, 2H), 4.41 – 4.50 (m, 2H, CH_2), 5.05 (dd, $J = 12.8, 5.4$ Hz, 1H, 3'-H), 6.09 (s, 1H, OH), 6.60 (t, $J = 5.8$ Hz, 1H, CH_2NH), 6.94 (s, 1H, $\text{CH}_{\text{arom. indacene}}$), 7.04 (d, $J = 7.0$ Hz, 1H), 7.14 (d, $J = 8.6$ Hz, 1H, 5-H, 7-H), 7.58 (dd, $J = 8.4, 7.2$ Hz, 1H, 6-H), 7.71 (s, 1H), 7.74 (s, 1H, $\text{CH}_{\text{arom. thiophene}}$), 7.87 (s, 1H, $\text{CH}_{\text{arom. triazole}}$), 8.11 (s, 1H, CONH), 10.87 (s, 1H, SO_2NH), 11.07 (s, 1H, NH imide); ^{13}C NMR (151 MHz, $\text{DMSO}-d_6$) δ 22.1, 25.0, 25.5, 29.2, 30.1, 30.6, 31.0, 32.4, 41.7, 48.5, 49.3, 68.6, 68.9, 69.4, 69.5, 69.6, 69.7, 69.7, 69.8, 69.8, 69.8, 70.2, 109.2, 110.6, 117.4, 118.0, 121.6, 127.5, 128.7, 132.0, 132.1, 136.2, 137.2, 143.1, 146.4, 149.9, 153.9, 167.3, 168.9, 170.0, 172.7; LC-MS (ESI) (90% H_2O to 100% MeCN in 10 min, then 100% MeCN to 15 min, DAD 200-600 nm), $t_R = 5.93$ min, 97% purity, m/z $[\text{M} + \text{H}]^+$ calcd for $\text{C}_{56}\text{H}_{76}\text{N}_8\text{O}_{16}\text{S}_2$ 1181.49, found 1181.8; HRMS (ESI) m/z $[\text{M} + \text{H}]^+$ calcd for $\text{C}_{56}\text{H}_{76}\text{N}_8\text{O}_{16}\text{S}_2$ 1181.4893, found 1181.4895.

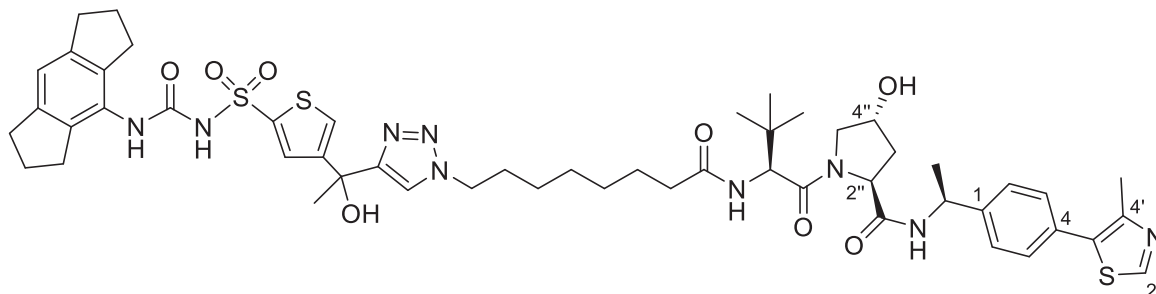
(2*S*,4*R*)-1-((2*S*)-2-(6-(4-(1-(5-(*N*-((1,2,3,5,6,7-Hexahydro-*s*-indacen-4-yl)carbamoyl)sulfamoyl)thiophen-3-yl)-1-hydroxyethyl)-1*H*-1,2,3-triazol-1-yl)hexanamido)-3,3-dimethylbutanoyl)-4-hydroxy-*N*-((*S*)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)pyrrolidine-2-carboxamide (141)



Compound **141** was synthesized following the general procedure for CuAAC and using azido-precursor **108** (200 μ mol). The crude product was purified by silica gel column chromatography using a CH₂Cl₂/MeOH gradient (0% to 20% MeOH) to yield a white solid (121 mg).

Yield 60%; mp: 135-141 °C; ¹H NMR (600 MHz, DMSO-*d*₆) δ 0.93 (s, 9H, C(CH₃)₃), 1.19 – 1.24 (m, 2H, CH₂), 1.37 (d, *J* = 7.0 Hz, 3H, CHCH₃), 1.46 – 1.55 (m, 2H, CH₂), 1.74 – 1.83 (m, 6H, CH₂, 3'-H, CH₃CO), 1.93 (quint, *J* = 7.3 Hz, 4H, CH₂ indacene), 1.98 – 2.03 (m, 1H, 3''-H), 2.08 – 2.15 (m, 1H), 2.20 – 2.27 (m, 1H, CH₂CON), 2.45 (s, 3H, CH₃ thiazole), 2.58 (t, *J* = 7.4 Hz, 4H, CH₂ indacene), 2.79 (t, *J* = 7.4 Hz, 4H, CH₂ indacene), 3.55 – 3.65 (m, 2H, 5''-H), 4.22 – 4.31 (m, 3H), 4.42 (t, *J* = 8.0 Hz, 1H), 4.51 (d, *J* = 9.3 Hz, 1H, 2''-H, 4''-H, NHCH₂, CH₂), 4.92 (quint, *J* = 7.2 Hz, 1H, CHCH₃), 5.08 (d, *J* = 2.8 Hz, 1H, CHO_H), 6.08 (s, 1H, COH), 6.94 (s, 1H, CH_{arom.} indacene), 7.33 – 7.40 (m, 2H), 7.41 – 7.46 (m, 2H, 2-H, 3-H, 5-H, 6-H), 7.70 – 7.73 (m, 1H), 7.73 – 7.76 (m, 1H, CH_{arom.} thiophene), 7.78 (d, *J* = 9.3 Hz, 1H, CONH), 7.89 (s, 1H, CH_{arom.} triazole), 8.11 (s, 1H, NHCON), 8.34 (d, *J* = 7.8 Hz, 1H, CONH), 8.98 (s, 1H, 2'-H), 10.91 (s, 1H, SO₂NH); ¹³C NMR (151 MHz, DMSO-*d*₆) δ 15.9, 22.4, 24.8, 25.0, 25.5, 26.4, 29.4, 30.1, 30.7, 32.4, 34.6, 35.1, 37.7, 47.7, 49.2, 56.2, 56.3, 58.5, 68.7, 69.5, 118.0, 121.1, 126.3, 127.7, 128.5, 128.8, 129.7, 131.1, 132.1, 137.2, 140.0, 143.1, 144.6, 147.7, 148.9, 149.9, 151.4, 153.9, 169.6, 170.6, 171.8; LC-MS (ESI) (90% H₂O to 100% MeCN in 10 min, then 100% MeCN to 15 min, DAD 200-600 nm), *t*_R = 5.60 min, 97% purity, *m/z* [M + H]⁺ calcd for C₅₀H₆₃N₉O₈S₃ 1014.40, found 1014.7; HRMS (ESI) *m/z* [M + H]⁺ calcd for C₅₀H₆₃N₉O₈S₃ 1014.4034, found 1014.4036.

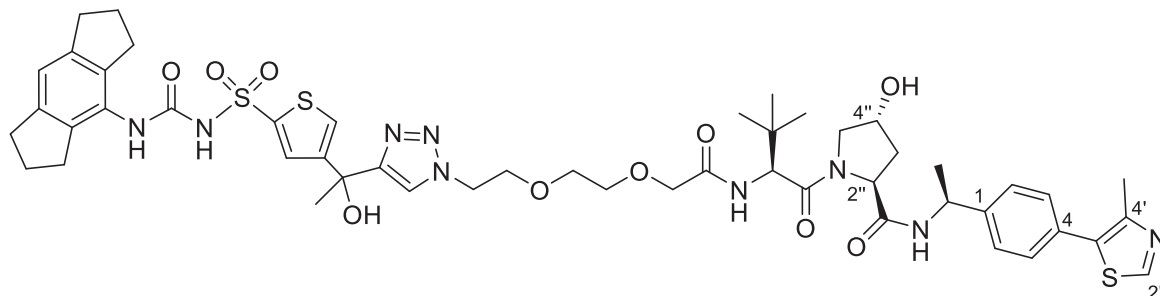
(2*S*,4*R*)-1-((2*S*)-2-(8-(4-(1-(5-(*N*-((1,2,3,5,6,7-Hexahydro-*s*-indacen-4-yl)carbamoyl)sulfamoyl)thiophen-3-yl)-1-hydroxyethyl)-1*H*-1,2,3-triazol-1-yl)octanamido)-3,3-dimethylbutanoyl)-4-hydroxy-*N*-((*S*)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)pyrrolidine-2-carboxamide (142)



Compound **142** was synthesized following the general procedure for CuAAC and using azido-precursor **109** (200 μ mol). The crude product was purified by silica gel column chromatography using a CH₂Cl₂/MeOH gradient (0% to 20% MeOH) to yield a white solid (93 mg).

Yield 45%; mp: 126-132 °C; ¹H NMR (600 MHz, DMSO-*d*₆) δ 0.93 (s, 9H, C(CH₃)₃), 1.14 – 1.25 (m, 6H, CH₂), 1.37 (d, *J* = 7.0 Hz, 3H, CHCH₃), 1.41 – 1.52 (m, 2H, CH₂), 1.72 – 1.83 (m, 6H, CH₂, 3''-H, CH₃CO), 1.93 (quint, *J* = 7.4 Hz, 4H, CH₂ indacene), 1.98 – 2.03 (m, 1H, 3''-H), 2.07 – 2.13 (m, 1H), 2.20 – 2.27 (m, 1H, CH₂CON), 2.45 (s, 3H, CH₃ thiazole), 2.58 (t, *J* = 7.4 Hz, 4H, CH₂ indacene), 2.78 (t, *J* = 7.4 Hz, 4H, CH₂ indacene), 3.55 – 3.66 (m, 2H, 5''-H), 4.22 – 4.31 (m, 3H), 4.42 (t, *J* = 8.0 Hz, 1H), 4.52 (d, *J* = 9.3 Hz, 1H, 2''-H, 4''-H, NHCH₂, CH₂), 4.89 – 4.95 (m, 1H, CHCH₃), 5.08 (d, *J* = 3.5 Hz, 1H, CHO), 6.07 (s, 1H, COH), 6.93 (s, 1H, CH_{arom.} indacene), 7.38 (d, *J* = 8.2 Hz, 2H), 7.42 – 7.45 (m, 2H, 2-H, 3-H, 5-H, 6-H), 7.70 (s, 1H), 7.71 – 7.78 (m, 2H, CH_{arom.} thiophene, CONH), 7.88 (s, 1H, CH_{arom.} triazole), 8.02 – 8.17 (m, 1H, NHCON), 8.34 (d, *J* = 7.8 Hz, 1H, CONH), 8.98 (s, 1H, 2'-H), 10.87 (s, 1H, SO₂NH); ¹³C NMR (151 MHz, DMSO-*d*₆) δ 16.0, 22.4, 25.0, 25.3, 25.8, 26.4, 28.1, 28.4, 29.7, 30.1, 30.6, 32.4, 34.8, 35.2, 37.7, 47.7, 49.2, 56.2, 56.3, 58.5, 68.7, 69.5, 117.9, 121.2, 126.4, 127.4, 128.8, 129.7, 131.1, 131.9, 137.2, 140.4, 143.0, 144.6, 147.7, 149.9, 151.4, 153.9, 169.6, 170.6, 172.0; LC-MS (ESI) (90% H₂O to 100% MeCN in 10 min, then 100% MeCN to 15 min, DAD 200-600 nm), *t*_R = 5.76 min, 97% purity, *m/z* [M + H]⁺ calcd for C₅₂H₆₇N₉O₈S₃ 1042.43, found 1042.7; HRMS (ESI) *m/z* [M + H]⁺ calcd for C₅₂H₆₇N₉O₈S₃ 1042.4347, found 1042.4346.

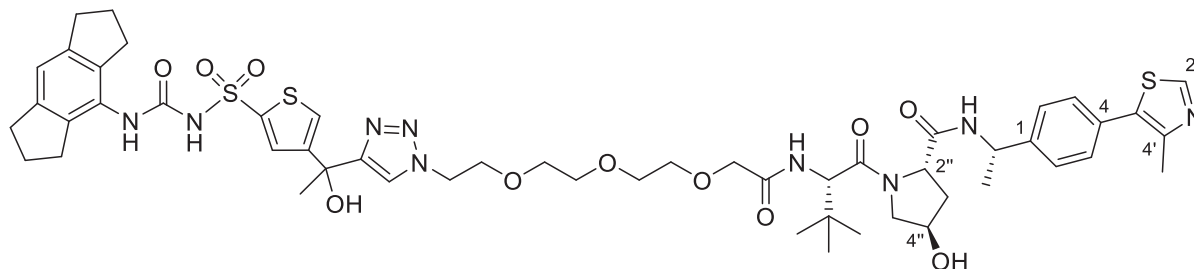
(2*S*,4*R*)-1-((2*S*)-2-(2-(2-(2-(4-(1-(5-(*N*-((1,2,3,5,6,7-hexahydro-*s*-indacen-4-yl)carbamoyl)sulfamoyl)thiophen-3-yl)-1-hydroxyethyl)-1*H*-1,2,3-triazol-1-yl)ethoxy)ethoxy)acetamido)-3,3-dimethylbutanoyl)-4-hydroxy-*N*-((*S*)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)pyrrolidine-2-carboxamide (143)



Compound **143** was synthesized following the general procedure for CuAAC and using azido-precursor **110** (200 μ mol). The crude product was purified by silica gel column chromatography using a CH₂Cl₂/MeOH gradient (0% to 20% MeOH) to yield a white solid (112 mg).

Yield 54%; mp: 127-133 °C; ¹H NMR (600 MHz, DMSO-*d*₆) δ 0.93 (s, 9H, C(CH₃)₃), 1.31 – 1.38 (m, 3H, CHCH₃), 1.75 – 1.81 (m, 4H, 3''-H, CH₃CO), 1.93 (quint, *J* = 7.3 Hz, 4H, CH₂ indacene), 2.02 – 2.08 (m, 1H, 3''-H), 2.45 (s, 3H, CH₃ thiazole), 2.59 (t, *J* = 7.4 Hz, 4H, CH₂ indacene), 2.78 (t, *J* = 7.4 Hz, 4H, CH₂ indacene), 3.52 – 3.63 (m, 6H, 5''-H, CH₂), 3.79 – 3.86 (m, 2H, CH₂), 3.87 – 3.97 (m, 2H, CH₂CON), 4.27 – 4.31 (m, 1H), 4.42 – 4.47 (m, 1H), 4.48 – 4.53 (m, 2H), 4.55 (d, *J* = 9.5 Hz, 1H, 2''-H, 4''-H, NHCH, CH₂), 4.87 – 4.93 (m, 1H, CHCH₃), 5.12 (d, *J* = 2.8 Hz, 1H, CHOH), 6.10 (s, 1H, COH), 6.94 (s, 1H, CH_{arom.} indacene), 7.33 – 7.45 (m, 5H, 2-H, 3-H, 5-H, 6-H, CONH), 7.70 – 7.74 (m, 1H), 7.74 – 7.77 (m, 1H, CH_{arom.} thiophene), 7.89 – 7.92 (m, 1H, CH_{arom.} triazole), 8.11 (s, 1H, NHCON), 8.36 – 8.45 (m, 1H, CONH), 8.98 (s, 1H, 2'-H), 10.87 (s, 1H, SO₂NH); ¹³C NMR (151 MHz, DMSO-*d*₆) δ 16.0, 22.4, 25.0, 26.2, 30.1, 30.6, 32.4, 35.8, 37.7, 47.7, 49.2, 55.7, 56.5, 58.6, 68.8, 68.8, 69.4, 69.5, 69.6, 70.3, 118.0, 121.7, 126.3, 127.6, 128.6, 128.8, 129.7, 131.1, 132.0, 137.2, 140.1, 143.1, 144.6, 147.7, 149.9, 151.4, 153.9, 168.5, 169.0, 170.4; LC-MS (ESI) (90% H₂O to 100% MeCN in 10 min, then 100% MeCN to 15 min, DAD 200-600 nm), *t*_R = 5.50 min, 96% purity, *m/z* [M + H]⁺ calcd for C₅₀H₆₃N₉O₁₀S₃ 1046.39, found 1046.7; HRMS (ESI) *m/z* [M + H]⁺ calcd for C₅₀H₆₃N₉O₁₀S₃ 1046.3933, found 1046.3933.

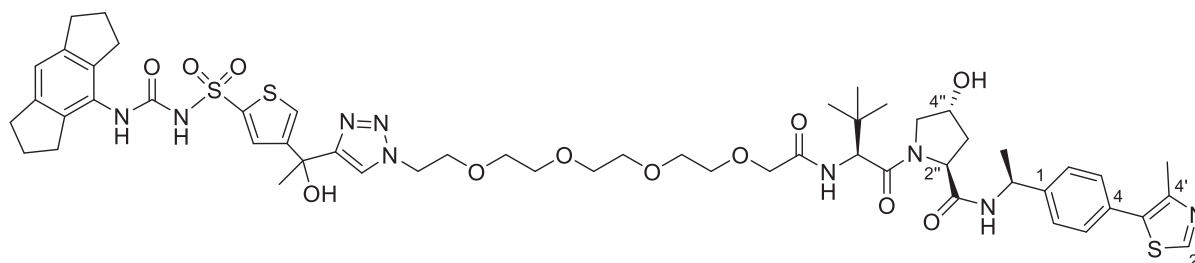
(2*S*,4*R*)-1-((2*S*)-2-(*tert*-Butyl)-14-(4-(1-(5-(*N*-((1,2,3,5,6,7-hexahydro-*s*-indacen-4-yl)carbamoyl)sulfamoyl)thiophen-3-yl)-1-hydroxyethyl)-1*H*-1,2,3-triazol-1-yl)-4-oxo-6,9,12-trioxa-3-azatetradecanoyl)-4-hydroxy-*N*-((*S*)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)pyrrolidine-2-carboxamide (144)



Compound **144** was synthesized following the general procedure for CuAAC and using azido-precursor **111** (200 μ mol). The crude product was purified by silica gel column chromatography using a CH₂Cl₂/MeOH gradient (0% to 20% MeOH) to yield a slightly beige solid (63 mg).

Yield 29%; mp: 113-119 °C; ¹H NMR (600 MHz, DMSO-*d*₆) δ 0.94 (s, 9H, C(CH₃)₃), 1.36 (d, *J* = 7.0 Hz, 3H, CHCH₃), 1.74 – 1.81 (m, 4H, 3''-H, CH₃CO), 1.93 (quint, *J* = 7.4 Hz, 4H, CH₂ indacene), 2.02 – 2.08 (m, 1H, 3''-H), 2.45 (s, 3H, CH₃ thiazole), 2.59 (t, *J* = 7.4 Hz, 4H, CH₂ indacene), 2.78 (t, *J* = 7.4 Hz, 4H, CH₂ indacene), 3.49 – 3.55 (m, 6H), 3.55 – 3.62 (m, 4H, 5''-H, CH₂), 3.80 (t, *J* = 5.4 Hz, 2H, CH₂), 3.95 (s, 2H, CH₂CON), 4.26 – 4.31 (m, 1H), 4.43 – 4.49 (m, 3H), 4.55 (d, *J* = 9.5 Hz, 1H, 2''-H, 4''-H, NHCH₂, CH₂), 4.87 – 4.93 (m, 1H, CHCH₃), 5.11 (d, *J* = 3.4 Hz, 1H, CHO_H), 6.10 (s, 1H, COH), 6.93 (s, 1H, CH_{arom.} indacene), 7.33 – 7.39 (m, 3H), 7.41 – 7.45 (m, 2H, 2-H, 3-H, 5-H, 6-H, CONH), 7.71 (s, 1H), 7.74 (s, 1H, CH_{arom.} thiophene), 7.88 (s, 1H, CH_{arom.} triazole), 8.10 (s, 1H, NHCON), 8.41 (d, *J* = 7.7 Hz, 1H, CONH), 8.98 (s, 1H, 2'-H), 10.86 (s, 1H, SO₂NH); ¹³C NMR (151 MHz, DMSO-*d*₆) δ 16.0, 22.4, 25.0, 26.2, 30.1, 30.6, 32.4, 35.7, 37.7, 47.7, 49.3, 55.7, 56.5, 58.5, 68.7, 68.7, 69.5, 69.6, 69.6, 69.7, 70.4, 117.9, 121.6, 126.3, 127.4, 128.8, 129.7, 131.1, 131.9, 137.2, 143.0, 144.7, 147.7, 149.9, 151.5, 153.9, 168.5, 169.0, 170.4; LC-MS (ESI) (90% H₂O to 100% MeCN in 10 min, then 100% MeCN to 15 min, DAD 200-600 nm), *t*_R = 5.64 min, 97% purity, *m/z* [M + H]⁺ calcd for C₅₂H₆₇N₉O₁₁S₃ 1090.42, found 1090.7; HRMS (ESI) *m/z* [M + Na]⁺ calcd for C₅₂H₆₇N₉O₁₁S₃ 1112.4014, found 1112.4067.

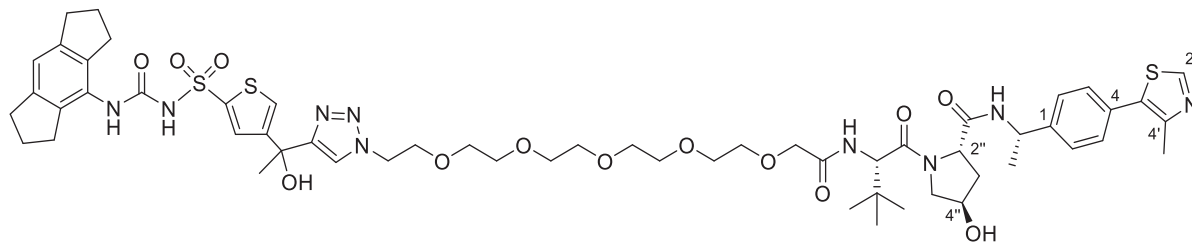
(2*S*,4*R*)-1-((2*S*)-2-(*tert*-Butyl)-17-(4-(1-(5-(*N*-((1,2,3,5,6,7-hexahydro-*s*-indacen-4-yl)carbamoyl)sulfamoyl)thiophen-3-yl)-1-hydroxyethyl)-1*H*-1,2,3-triazol-1-yl)-4-oxo-6,9,12,15-tetraoxa-3-azaheptadecanoyl)-4-hydroxy-*N*-((*S*)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)pyrrolidine-2-carboxamide (145)



Compound **145** was synthesized following the general procedure for CuAAC and using azido-precursor **112** (200 μ mol). The crude product was purified by silica gel column chromatography using a CH₂Cl₂/MeOH gradient (0% to 20% MeOH) to yield a white solid (106 mg).

Yield 47%; mp: 107-113 °C; ¹H NMR (600 MHz, DMSO-*d*₆) δ 0.94 (s, 9H, C(CH₃)₃), 1.37 (d, *J* = 6.9 Hz, 3H, CHCH₃), 1.73 – 1.84 (m, 4H, 3''-H, CH₃CO), 1.93 (quint, *J* = 7.4 Hz, 4H, CH₂ indacene), 2.02 – 2.08 (m, 1H, 3''), 2.45 (s, 3H, CH₃ thiazole), 2.58 (t, *J* = 7.4 Hz, 4H, CH₂ indacene), 2.78 (t, *J* = 7.4 Hz, 4H, CH₂ indacene), 3.45 – 3.63 (m, 14H, 5''-H, CH₂), 3.79 (t, *J* = 5.3 Hz, 2H, CH₂), 3.92 – 3.99 (m, 2H, CH₂CON), 4.25 – 4.31 (m, 1H), 4.41 – 4.50 (m, 3H), 4.55 (d, *J* = 9.6 Hz, 1H, 2''-H, 4''-H, NHCH, CH₂), 4.87 – 4.94 (m, 1H, CHCH₃), 5.11 (d, *J* = 3.0 Hz, 1H, CHOH), 6.10 (s, 1H, COH), 6.94 (s, 1H, CH_{arom.} indacene), 7.33 – 7.40 (m, 3H), 7.41 – 7.46 (m, 2H, 2-H, 3-H, 5-H, 6-H, CONH), 7.70 – 7.73 (m, 1H), 7.73 – 7.77 (m, 1H, CH_{arom.} thiophene), 7.88 (s, 1H, CH_{arom.} triazole), 8.12 (s, 1H, NHCON), 8.41 (d, *J* = 7.7 Hz, 1H, CONH), 8.98 (s, 1H, 2'-H), 10.85 (s, 1H, SO₂NH); ¹³C NMR (151 MHz, DMSO-*d*₆) δ 16.0, 22.4, 25.0, 26.2, 30.1, 30.6, 32.4, 35.7, 37.7, 47.7, 49.3, 55.7, 56.5, 58.5, 68.6, 68.7, 69.5, 69.6, 69.6, 69.6, 69.7, 69.8, 70.4, 118.0, 121.6, 126.3, 127.6, 128.6, 128.8, 129.7, 131.1, 132.0, 137.2, 143.1, 144.7, 147.8, 149.9, 151.5, 153.9, 168.5, 169.0, 170.4; LC-MS (ESI) (90% H₂O to 100% MeCN in 10 min, then 100% MeCN to 15 min, DAD 200-600 nm), *t*_R = 5.49 min, 95% purity, *m/z* [M + H]⁺ calcd for C₅₄H₇₁N₉O₁₂S₃ 1134.45, found 1134.7; HRMS (ESI) *m/z* [M + H]⁺ calcd for C₅₄H₇₁N₉O₁₂S₃ 1134.4457, found 1134.4444.

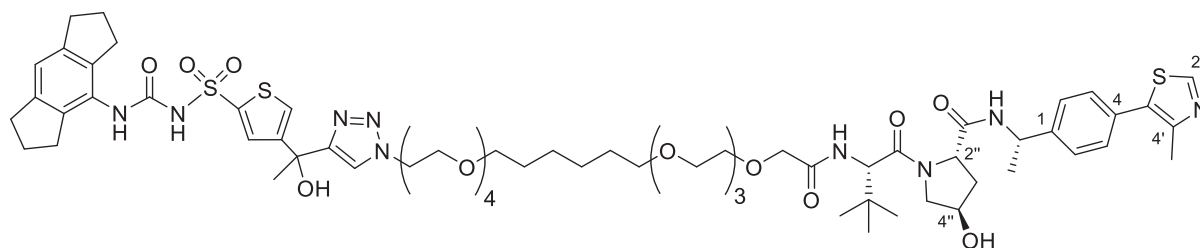
(2*S*,4*R*)-1-((2*S*)-2-(*tert*-Butyl)-20-(4-(1-(5-(*N*-((1,2,3,5,6,7-hexahydro-*s*-indacen-4-yl)carbamoyl)sulfamoyl)thiophen-3-yl)-1-hydroxyethyl)-1*H*-1,2,3-triazol-1-yl)-4-oxo-6,9,12,15,18-pentaoxa-3-azaicosanoyl)-4-hydroxy-*N*-((*S*)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)pyrrolidine-2-carboxamide (146)



Compound **146** was synthesized following the general procedure for CuAAC and using azido-precursor **113** (200 μ mol). The crude product was purified by silica gel column chromatography using a CH₂Cl₂/MeOH gradient (0% to 20% MeOH) to yield a white solid (100 mg).

Yield 42%; mp: 92-98 °C; ¹H NMR (600 MHz, DMSO-*d*₆) δ 0.94 (s, 9H, C(CH₃)₃), 1.37 (d, J = 7.0 Hz, 3H, CHCH₃), 1.75 – 1.81 (m, 4H, 3''-H, CH₃CO), 1.93 (quint, J = 7.4 Hz, 4H, CH₂ indacene), 2.02 – 2.07 (m, 1H, 3''-H), 2.45 (s, 3H, CH₃ thiazole), 2.58 (t, J = 7.4 Hz, 4H, CH₂ indacene), 2.78 (t, J = 7.4 Hz, 4H, CH₂ indacene), 3.45 – 3.54 (m, 12H), 3.55 – 3.63 (m, 6H, 5''-H, CH₂), 3.79 (t, J = 5.3 Hz, 2H, CH₂), 3.96 (s, 2H, CH₂CON), 4.26 – 4.31 (m, 1H), 4.42 – 4.48 (m, 3H), 4.54 (d, J = 9.5 Hz, 1H, 2''-H, 4''-H, NHCH, CH₂), 4.91 (quint, J = 7.1 Hz, 1H, CHCH₃), 5.11 (d, J = 2.9 Hz, 1H, CHOH), 6.10 (s, 1H, COH), 6.94 (s, 1H, CH_{arom.} indacene), 7.32 – 7.40 (m, 3H), 7.41 – 7.46 (m, 2H, 2-H, 3-H, 5-H, 6-H, CONH), 7.70 – 7.73 (m, 1H), 7.73 – 7.76 (m, 1H, CH_{arom.} thiophene), 7.88 (s, 1H, CH_{arom.} triazole), 8.11 (s, 1H, NHCON), 8.41 (d, J = 7.7 Hz, 1H, CONH), 8.98 (s, 1H, 2'-H), 10.91 (s, 1H, SO₂NH); ¹³C NMR (151 MHz, DMSO-*d*₆) δ 15.9, 22.4, 25.0, 26.2, 30.1, 30.6, 32.4, 35.7, 37.7, 47.7, 49.3, 55.7, 56.5, 58.5, 68.6, 68.7, 69.5, 69.6, 69.6, 69.7, 69.8, 70.4, 118.0, 121.6, 126.3, 127.6, 128.6, 128.8, 129.7, 131.1, 132.1, 137.2, 140.0, 143.1, 144.7, 147.7, 148.9, 149.9, 151.4, 153.9, 168.5, 169.0, 170.4; LC-MS (ESI) (90% H₂O to 100% MeCN in 10 min, then 100% MeCN to 15 min, DAD 200-600 nm), t_R = 5.48 min, 98% purity, m/z [M + H]⁺ calcd for C₅₆H₇₅N₉O₁₃S₃ 1178.47, found 1178.8; HRMS (ESI) m/z [M + H]⁺ calcd for C₅₆H₇₅N₉O₁₃S₃ 1178.4719, found 1178.4706.

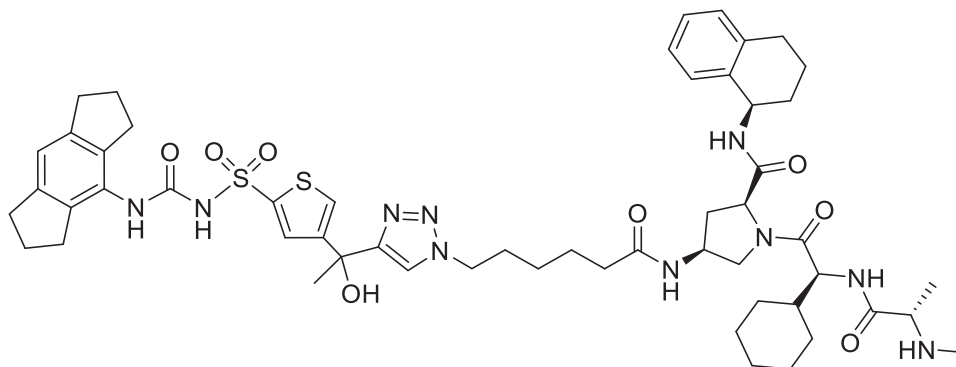
(2*S*,4*R*)-1-((2*S*)-2-(*tert*-Butyl)-33-(4-(1-(5-(*N*-((1,2,3,5,6,7-hexahydro-*s*-indacen-4-yl)carbamoyl)sulfamoyl)thiophen-3-yl)-1-hydroxyethyl)-1*H*-1,2,3-triazol-1-yl)-4-oxo-6,9,12,15,22,25,28,31-octaoxa-3-azatritriacontanoyl)-4-hydroxy-*N*-((*S*)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)pyrrolidine-2-carboxamide (147)



Compound **147** was synthesized following the general procedure for CuAAC and using azido-precursor **114** (150 μ mol). The crude product was purified by silica gel column chromatography using a CH₂Cl₂/MeOH gradient (0% to 20% MeOH). The obtained product was further purified by C18-reversed phase silica gel flash column chromatography using a H₂O/MeCN gradient (20% to 100% MeCN) to yield a white solid (32 mg).

Yield 16%; mp: 76-82 °C; ¹H NMR (600 MHz, DMSO-*d*₆) δ 0.94 (s, 9H, C(CH₃)₃), 1.25 – 1.30 (m, 4H, CH₂), 1.38 (d, *J* = 7.0 Hz, 3H, CHCH₃), 1.43 – 1.50 (m, 4H, CH₂), 1.73 – 1.81 (m, 4H, 3''-H, CH₃CO), 1.86 – 1.92 (m, 4H, CH₂ indacene), 2.01 – 2.07 (m, 1H, 3''-H), 2.45 (s, 3H, CH₃ thiazole), 2.63 – 2.69 (m, 4H, CH₂ indacene), 2.71 – 2.77 (m, 4H, CH₂ indacene), 3.33 – 3.38 (m, 4H), 3.43 – 3.64 (m, 26H, 5''-H, CH₂), 3.74 – 3.82 (m, 2H, CH₂), 3.96 (s, 2H, CH₂CON), 4.28 (s, 1H), 4.40 – 4.49 (m, 3H), 4.54 (d, *J* = 9.4 Hz, 1H, 2''-H, 4''-H, NHCH₂, CH₂), 4.87 – 4.94 (m, 1H, CHCH₃), 5.11 (s, 1H, CHOH), 5.82 (s, 1H, COH), 6.76 (s, 1H), 7.18 (s, 1H), 7.27 – 7.39 (m, 4H), 7.39 – 7.51 (m, 3H), 7.80 (s, 1H, CH_{arom.}, CONH, NHCON), 8.41 (d, *J* = 7.7 Hz, 1H, CONH), 8.98 (s, 1H, 2'-H), one signal (1H, SO₂NH) is missing due to proton exchange; ¹³C NMR (151 MHz, DMSO-*d*₆) δ 15.9, 22.4, 25.1, 25.5, 26.2, 29.2, 30.4, 30.6, 32.6, 35.7, 37.7, 47.7, 49.2, 55.7, 56.5, 58.5, 68.7, 68.7, 69.4, 69.5, 69.6, 69.6, 69.7, 69.8, 69.8, 70.2, 70.4, 115.4, 121.5, 126.3, 128.8, 129.7, 131.1, 136.4, 142.1, 144.7, 147.7, 148.5, 149.6, 151.4, 154.4, 168.5, 169.0, 170.4; LC-MS (ESI) (90% H₂O to 100% MeCN in 10 min, then 100% MeCN to 15 min, DAD 200-600 nm), *t*_R = 6.07 min, 96% purity, *m/z* [M + H]⁺ calcd for C₆₆H₉₅N₉O₁₆S₃ 1366.61, found 1366.9; HRMS (ESI) *m/z* [M + H]⁺ calcd for C₆₆H₉₅N₉O₁₆S₃ 1366.6132, found 1366.6142.

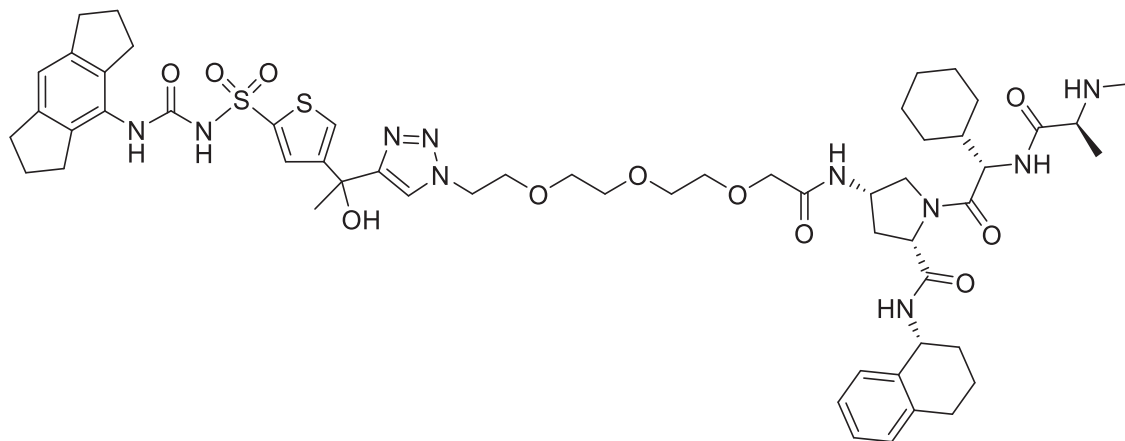
(2*S*,4*S*)-1-((*S*)-2-Cyclohexyl-2-((*S*)-2-(methylamino)propanamido)acetyl)-4-(6-(4-(1-(5-(*N*-((1,2,3,5,6,7-hexahydro-*s*-indacen-4-yl)carbamoyl)sulfamoyl)thiophen-3-yl)-1-hydroxyethyl)-1*H*-1,2,3-triazol-1-yl)hexanamido)-*N*-((*R*)-1,2,3,4-tetrahydronaphthalen-1-yl)pyrrolidine-2-carboxamide (148)



Compound **115** (145 mg, 200 μmol) was dissolved in dry CH_2Cl_2 (2 mL), treated with TFA (2 mL) and stirred for 2 h at room temperature. The mixture was concentrated *in vacuo*. Compound **148** was then synthesized following the general procedure for CuAAC and using the deprotected azido-precursor of **115**. The crude product was purified by silica gel flash column chromatography using a $\text{CH}_2\text{Cl}_2/\text{MeOH}$ gradient (0% to 20% MeOH) to yield a white solid (23 mg).

Yield 11%; mp: 145-151 $^\circ\text{C}$; $^1\text{H NMR}$ (600 MHz, $\text{DMSO}-d_6$) δ 0.94 – 1.30 (m, 8H), 1.31 (d, J = 6.9 Hz, 3H), 1.47 – 1.54 (m, 2H), 1.59 – 1.81 (m, 13H), 1.81 – 1.97 (m, 7H), 2.01 – 2.09 (m, 2H), 2.33 – 2.39 (m, 1H), 2.48 (s, 3H), 2.61 (t, J = 7.5 Hz, 4H), 2.66 – 2.75 (m, 2H), 2.77 (t, J = 7.4 Hz, 4H), 3.79 – 3.84 (m, 1H), 4.08 – 4.14 (m, 1H), 4.19 – 4.33 (m, 4H), 4.37 – 4.41 (m, 1H), 4.90 – 4.97 (m, 1H), 5.95 (s, 1H), 6.86 (s, 1H), 7.05 – 7.12 (m, 2H), 7.12 – 7.17 (m, 1H), 7.29 (d, J = 7.5 Hz, 1H), 7.48 (s, 1H), 7.57 (s, 1H), 7.85 (s, 1H), 7.87 – 7.95 (m, 1H), 8.12 (d, J = 7.5 Hz, 1H), 8.35 – 8.43 (m, 1H), 8.72 (d, J = 8.0 Hz, 1H), two signals (2H) are missing due to proton exchange; $^{13}\text{C NMR}$ (151 MHz, $\text{DMSO}-d_6$) δ 15.7, 20.1, 24.4, 25.0, 25.5, 25.7, 25.8, 28.0, 28.1, 28.5, 28.7, 29.4, 29.8, 30.3, 30.5, 30.8, 32.5, 34.3, 35.1, 46.6, 47.6, 49.1, 52.2, 55.4, 55.9, 58.4, 69.5, 116.9, 121.1, 125.6, 126.6, 128.3, 128.5, 136.9, 137.0, 137.3, 142.6, 149.3, 154.1, 168.8, 169.2, 170.6, 171.9; **LC-MS** (ESI) (90% H_2O to 100% MeCN in 10 min, then 100% MeCN to 15 min, DAD 220-600 nm), t_R = 6.39 min, 97% purity, m/z $[\text{M} + \text{H}]^+$ calcd for $\text{C}_{54}\text{H}_{72}\text{N}_{10}\text{O}_8\text{S}_2$ 1053.50, found 1053.8; **HRMS** (ESI) m/z $[\text{M} + \text{H}]^+$ calcd for $\text{C}_{54}\text{H}_{72}\text{N}_{10}\text{O}_8\text{S}_2$ 1053.5049, found 1053.5052.

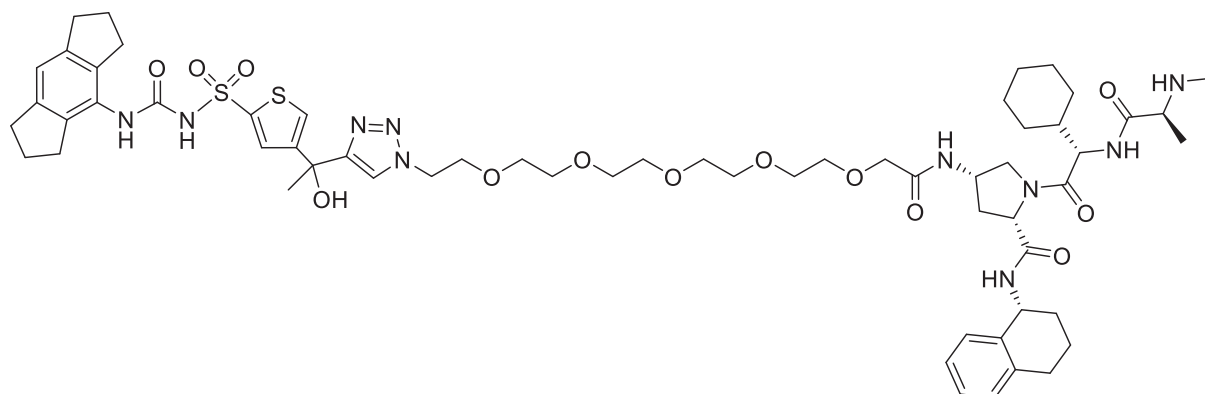
(2*S*,4*S*)-1-((*S*)-2-Cyclohexyl-2-((*S*)-2-(methylamino)propanamido)acetyl)-4-(2-(2-(2-(2-(4-(1-(5-(*N*-((1,2,3,5,6,7-hexahydro-*s*-indacen-4-yl)carbamoyl)sulfamoyl)thiophen-3-yl)-1-hydroxyethyl)-1*H*-1,2,3-triazol-1-yl)ethoxy)ethoxy)ethoxy)acetamido)-*N*-((*R*)-1,2,3,4-tetrahydronaphthalen-1-yl)pyrrolidine-2-carboxamide (149)



Compound **116** (160 mg, 200 μ mol) was dissolved in dry CH_2Cl_2 (2 mL), treated with TFA (2 mL) and stirred for 2 h at room temperature. The mixture was concentrated *in vacuo*. Compound **149** was then synthesized following the general procedure for CuAAC and using the deprotected azido-precursor of **116**. The crude product was purified by silica gel flash column chromatography using a $\text{CH}_2\text{Cl}_2/\text{MeOH}$ gradient (0% to 20% MeOH) to yield a white solid (32 mg).

Yield 14%; mp: 122-128 $^\circ\text{C}$; ^1H NMR (600 MHz, $\text{DMSO}-d_6$) δ 0.98 – 1.07 (m, 2H), 1.09 – 1.21 (m, 3H), 1.31 (d, J = 7.0 Hz, 3H), 1.56 – 1.75 (m, 7H), 1.78 (s, 3H), 1.80 – 1.99 (m, 8H), 2.33 – 2.41 (m, 1H), 2.53 – 2.66 (m, 4H), 2.66 – 2.83 (m, 6H), 3.47 – 3.61 (m, 10H), 3.75 – 3.93 (m, 5H), 4.00 – 4.07 (m, 1H), 4.28 – 4.50 (m, 5H), 4.92 – 4.98 (m, 1H), 6.00 (s, 1H), 6.88 (s, 1H), 7.07 – 7.11 (m, 2H), 7.13 – 7.18 (m, 1H), 7.29 (d, J = 6.9 Hz, 1H), 7.52 (s, 1H), 7.56 – 7.67 (m, 1H), 7.84 (s, 1H), 7.90 – 8.15 (m, 1H), 8.26 – 8.34 (m, 1H), 8.39 – 8.46 (m, 1H), 8.73 (d, J = 7.5 Hz, 1H), two signals (2H) are missing due to proton exchange, one signal (2H) is obscured by solvent signal; ^{13}C NMR (151 MHz, $\text{DMSO}-d_6$) δ 15.7, 20.2, 25.0, 25.5, 25.7, 25.8, 28.1, 28.4, 28.7, 29.7, 30.2, 30.5, 30.8, 32.5, 34.2, 46.7, 47.3, 49.2, 52.7, 55.4, 55.9, 58.5, 68.6, 69.2, 69.5, 69.6, 69.8, 69.9, 70.3, 117.1, 121.5, 125.6, 126.6, 128.3, 128.5, 137.0, 137.0, 137.3, 142.7, 149.4, 154.1, 168.7, 169.1, 169.3, 170.8; LC-MS (ESI) (90% H_2O to 100% MeCN in 10 min, then 100% MeCN to 15 min, DAD 220-600 nm), t_R = 6.20 min, 93% purity, m/z $[\text{M} + \text{H}]^+$ calcd for $\text{C}_{56}\text{H}_{76}\text{N}_{10}\text{O}_{11}\text{S}_2$ 1129.52, found 1129.9; HRMS (ESI) m/z $[\text{M} + \text{H}]^+$ calcd for $\text{C}_{56}\text{H}_{76}\text{N}_{10}\text{O}_{11}\text{S}_2$ 1129.5209, found 1129.5211.

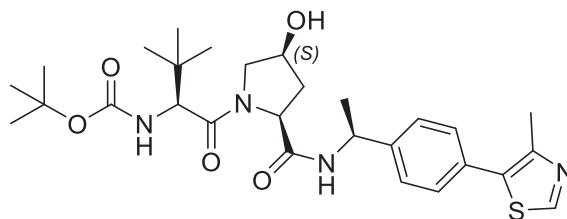
(2*S*,4*S*)-1-((*S*)-2-Cyclohexyl-2-((*S*)-2-(methylamino)propanamido)acetyl)-4-(17-(4-(1-(5-(*N*-((1,2,3,5,6,7-hexahydro-*s*-indacen-4-yl)carbamoyl)sulfamoyl)thiophen-3-yl)-1-hydroxyethyl)-1*H*-1,2,3-triazol-1-yl)-3,6,9,12,15-pentaoxaheptadecanamido)-*N*-((*R*)-1,2,3,4-tetrahydronaphthalen-1-yl)pyrrolidine-2-carboxamide (151)



Compound **118** (177 mg, 200 μ mol) was dissolved in dry CH_2Cl_2 (2 mL), treated with TFA (2 mL) and stirred for 2 h at room temperature. The mixture was concentrated *in vacuo*. Compound **151** was then synthesized following the general procedure for CuAAC and using the deprotected azido-precursor of **118**. The crude product was purified by silica gel flash column chromatography using a $\text{CH}_2\text{Cl}_2/\text{MeOH}$ gradient (0% to 25% MeOH) to yield a white solid (44 mg).

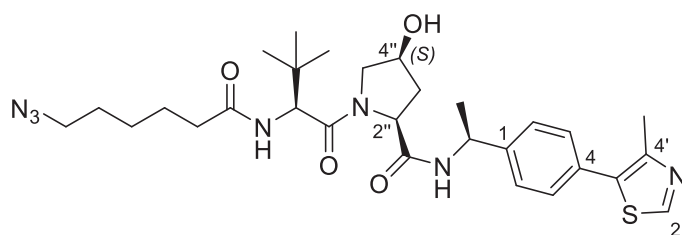
Yield 18%; mp: 118–124 $^\circ\text{C}$; ^1H NMR (600 MHz, $\text{DMSO}-d_6$) δ 0.97 – 1.09 (m, 2H), 1.09 – 1.23 (m, 3H), 1.29 – 1.37 (m, 3H), 1.57 – 1.77 (m, 7H), 1.79 (s, 3H), 1.80 – 1.96 (m, 8H), 2.33 – 2.40 (m, 1H), 2.59 (t, $J = 7.4$ Hz, 4H), 2.68 – 2.76 (m, 2H), 2.78 (t, $J = 7.4$ Hz, 4H), 3.41 – 3.63 (m, 18H), 3.78 (t, $J = 5.4$ Hz, 2H), 3.82 – 3.91 (m, 3H), 3.99 – 4.08 (m, 1H), 4.32 – 4.49 (m, 5H), 4.91 – 4.98 (m, 1H), 6.08 (s, 1H), 6.93 (s, 1H), 7.05 – 7.12 (m, 2H), 7.12 – 7.19 (m, 1H), 7.29 (d, $J = 7.5$ Hz, 1H), 7.69 (s, 1H), 7.73 (s, 1H), 7.87 (s, 1H), 8.25 (s, 1H), 8.31 (d, $J = 8.4$ Hz, 1H), 8.43 (d, $J = 8.7$ Hz, 1H), 8.75 (d, $J = 8.0$ Hz, 1H), two signals (2H) are missing due to proton exchange, one signal (2H) is obscured by solvent signal; ^{13}C NMR (151 MHz, $\text{DMSO}-d_6$) δ 15.6, 20.2, 25.0, 25.5, 25.7, 25.8, 28.1, 28.4, 28.7, 29.7, 30.1, 30.6, 30.9, 32.4, 34.2, 46.7, 47.3, 49.2, 52.8, 55.4, 55.8, 58.5, 68.6, 69.5, 69.6, 69.6, 69.7, 69.8, 69.9, 70.4, 117.9, 121.6, 125.6, 126.6, 127.4, 128.3, 128.5, 131.9, 136.9, 137.2, 137.3, 143.0, 149.9, 153.9, 168.6, 169.1, 169.3, 170.8; LC-MS (ESI) (90% H_2O to 100% MeCN in 10 min, then 100% MeCN to 15 min, DAD 220–600 nm), $t_R = 6.23$ min, 98% purity, m/z $[\text{M} + \text{H}]^+$ calcd for $\text{C}_{60}\text{H}_{84}\text{N}_{10}\text{O}_{13}\text{S}_2$ 1217.57, found 1217.9; HRMS (ESI) m/z $[\text{M} + \text{H}]^+$ calcd for $\text{C}_{60}\text{H}_{84}\text{N}_{10}\text{O}_{13}\text{S}_2$ 1217.5734, found 1217.5731.

tert-butyl ((*S*)-1-((2*S*,4*S*)-4-hydroxy-2-(((*S*)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)carbamoyl)pyrrolidin-1-yl)-3,3-dimethyl-1-oxobutan-2-yl)carbamate (**152**)



Compound **152** was prepared as described before.³²³

(2*S*,4*S*)-1-((*S*)-2-(6-Azidohexanamido)-3,3-dimethylbutanoyl)-4-hydroxy-*N*-((*S*)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)pyrrolidine-2-carboxamide (**153**)

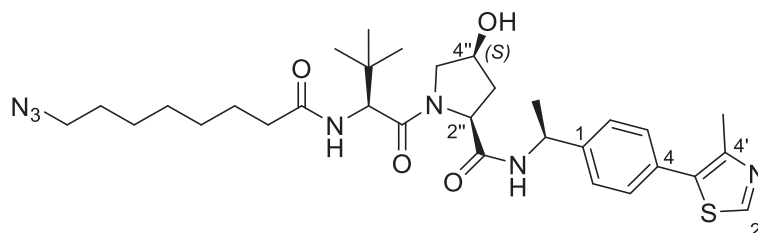


The Boc-protected (-)VHL ligand **152** (436 mg, 800 μ mol) was dissolved in dry CH_2Cl_2 (5 mL) and was treated with TFA (5 mL). The mixture was stirred for 2 h at room temperature. The mixture was concentrated *in vacuo*. 6-Azidohexanoic acid (**69**, 126 mg, 800 μ mol) was dissolved in dry DMF (5 mL). HATU (335 mg, 880 μ mol) and DIPEA (362 mg, 2.80 mmol) were added under argon. The deprotected (-)VHL ligand was dissolved in dry DMF (5 mL) and was added to the mixture, containing the activated acid compound. The reaction mixture was allowed to stir at room temperature under argon for 18 h. The reaction mixture was concentrated *in vacuo*. The crude product was purified by silica gel flash column chromatography using a $\text{CH}_2\text{Cl}_2/\text{MeOH}$ gradient (0% to 5% MeOH) as eluent to yield a colorless resin (295 mg).

Yield 63%; $^1\text{H NMR}$ (600 MHz, $\text{DMSO}-d_6$) δ 0.95 (s, 9H, $\text{C}(\text{CH}_3)_3$), 1.27 – 1.32 (m, 2H, CH_2), 1.38 (d, $J = 7.0$ Hz, 3H, CHCH_3), 1.43 – 1.55 (m, 4H, CH_2), 1.63 – 1.69 (m, 1H), 2.09 – 2.15 (m, 1H), 2.21 – 2.33 (m, 2H, 3''-H, CH_2CON), 2.46 (s, 3H, CH_3 thiazole), 3.39 (dd, $J = 10.1, 5.2$ Hz, 1H), 3.90 (dd, $J = 10.2, 5.6$ Hz, 1H, 5''-H), 4.16 – 4.22 (m, 1H), 4.33 (dd, $J = 8.7, 6.1$ Hz, 1H), 4.45 (d, $J = 8.8$ Hz, 1H, 2''-H, 4''-H, NHCH), 4.90 – 4.96 (m, 1H, CHCH_3), 5.31 (d, $J = 6.5$ Hz, 1H, OH), 7.35 – 7.41 (m, 2H), 7.41 – 7.47 (m, 2H, 2-H, 3-H, 5-H, 6-H), 7.81 (d, $J = 8.8$ Hz, 1H, CONH), 8.29 (d, $J = 7.7$ Hz, 1H, CONH), 8.98 (s, 1H, 2'-H), one signal (2H, CH_2N_3)

is obscured by solvent signal; ^{13}C NMR (151 MHz, DMSO- d_6) δ 15.9 (CH_3 thiazole), 22.2 (CHCH_3), 24.8, 25.7 (CH_2), 26.4 ($\text{C}(\text{CH}_3)_3$), 27.9 (CH_2), 34.5, 34.6, 36.8 (CH_2 , $\text{C}(\text{CH}_3)_3$, C-3''), 47.7 (CHCH_3), 50.5 (CH_2N_3), 55.5, 56.7, 58.5 (C-2'', C-5'', NHCH), 69.0 (C-4''), 126.4, 128.8 (C-2, C-3, C-5, C-6), 129.8, 131.1, 144.2 (C-1, C-4, C-5'), 147.8 (C-4'), 151.4 (C-2'), 169.9, 171.0, 172.2 (CO); LC-MS (ESI) (90% H_2O to 100% MeCN in 10 min, then 100% MeCN to 15 min, DAD 220-600 nm), t_{R} = 6.30 min, 98% purity, m/z $[\text{M} + \text{H}]^+$ calcd for $\text{C}_{29}\text{H}_{41}\text{N}_7\text{O}_4\text{S}$ 584.30, found 584.5.

(2*S*,4*S*)-1-((*S*)-2-(8-Azidoctanamido)-3,3-dimethylbutanoyl)-4-hydroxy-*N*-((*S*)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)pyrrolidine-2-carboxamide (154)

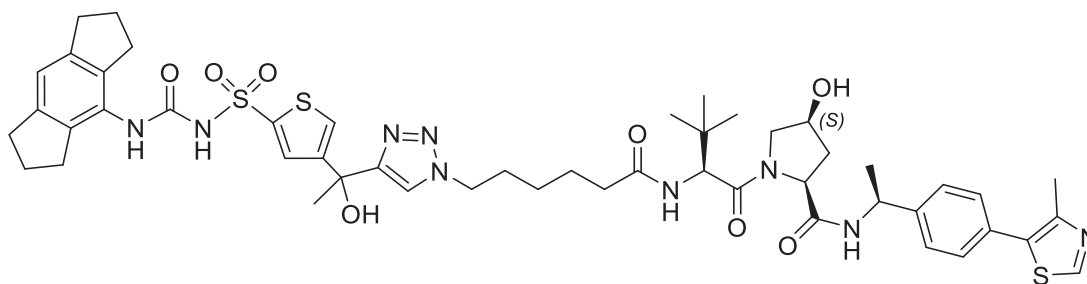


The Boc-protected (-)VHL ligand **152** (436 mg, 800 μmol) was dissolved in dry CH_2Cl_2 (5 mL) and was treated with TFA (5 mL). The mixture was stirred for 2 h at room temperature. The mixture was concentrated *in vacuo*. 8-Azidoctanoic acid (**71**, 148 mg, 800 μmol) was dissolved in dry DMF (5 mL). HATU (335 mg, 880 μmol) and DIPEA (362 mg, 2.80 mmol) were added under argon. The deprotected (-)VHL ligand was dissolved in dry DMF (5 mL) and was added to the mixture, containing the activated acid compound. The reaction mixture was allowed to stir at room temperature under argon for 18 h. The reaction mixture was concentrated *in vacuo*. The crude product was purified by silica gel flash column chromatography using a $\text{CH}_2\text{Cl}_2/\text{MeOH}$ gradient (0% to 5% MeOH) as eluent to yield a colorless resin (265 mg).

Yield 54%; ^1H NMR (500 MHz, DMSO- d_6) δ 0.95 (s, 9H, $\text{C}(\text{CH}_3)_3$), 1.17 – 1.33 (m, 6H, CH_2), 1.38 (d, J = 6.9 Hz, 3H, CHCH_3), 1.42 – 1.55 (m, 4H, CH_2), 1.63 – 1.69 (m, 1H), 2.06 – 2.14 (m, 1H), 2.20 – 2.27 (m, 1H), 2.27 – 2.34 (m, 1H, 3''-H, CH_2CON), 2.46 (s, 3H, CH_3 thiazole), 3.36 – 3.41 (m, 1H), 3.86 – 3.94 (m, 1H, 5''-H), 4.16 – 4.23 (m, 1H), 4.33 (dd, J = 8.7, 6.1 Hz, 1H), 4.45 (d, J = 8.8 Hz, 1H, 2''-H, 4''-H, NHCH), 4.89 – 4.96 (m, 1H, CHCH_3), 5.31 (d, J = 6.5 Hz, 1H, OH), 7.35 – 7.41 (m, 2H), 7.42 – 7.50 (m, 2H, 2-H, 3-H, 5-H, 6-H), 7.78 (d, J = 8.7 Hz, 1H, CONH), 8.29 (d, J = 7.7 Hz, 1H, CONH), 8.98 (s, 1H, 2'-H), one signal (2H, CH_2N_3) is obscured by solvent signal; ^{13}C NMR (126 MHz, DMSO- d_6) δ 15.9 (CH_3 thiazole), 22.1 (CHCH_3), 25.2, 26.0 (CH_2), 26.4 ($\text{C}(\text{CH}_3)_3$), 28.1, 28.4 (CH_2), 34.6, 34.7, 36.7 (CH_2 , $\text{C}(\text{CH}_3)_3$,

C-3''), 47.7 ($\underline{\text{CHCH}_3}$), 50.6 (CH_2N_3), 55.5, 56.6, 58.5 (C-2'', C-5'', NHCH), 68.9 (C-4''), 126.4, 128.8 (C-2, C-3, C-5, C-6), 129.7, 131.0, 144.2 (C-1, C-4, C-5'), 147.7 (C-4'), 151.4 (C-2'), 169.9, 171.0, 172.3 (CO), one signal (CH_2) is missing due to overlapping signals; **LC-MS** (ESI) (90% H_2O to 100% MeCN in 10 min, then 100% MeCN to 15 min, DAD 220-600 nm), $t_{\text{R}} = 7.12$ min, 95% purity, m/z $[\text{M} + \text{H}]^+$ calcd for $\text{C}_{31}\text{H}_{45}\text{N}_7\text{O}_4\text{S}$ 612.33, found 612.5.

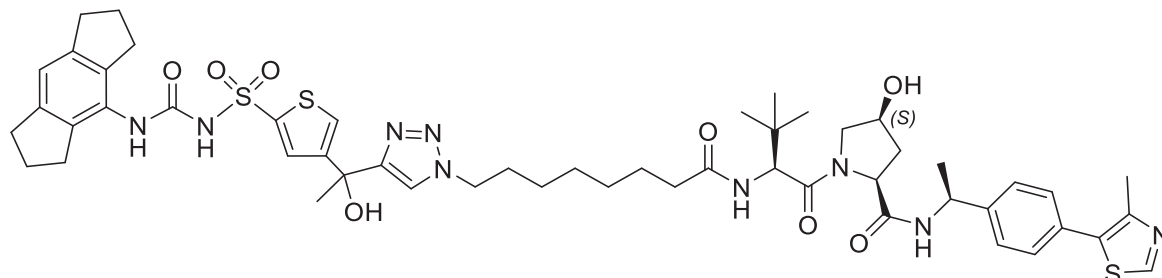
(2*S*,4*S*)-1-((2*S*)-2-(6-(4-(1-(5-(*N*-((1,2,3,5,6,7-Hexahydro-*s*-indacen-4-yl)carbamoyl)sulfamoyl)thiophen-3-yl)-1-hydroxyethyl)-1*H*-1,2,3-triazol-1-yl)hexanamido)-3,3-dimethylbutanoyl)-4-hydroxy-*N*-((*S*)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)pyrrolidine-2-carboxamide (155)



Compound **155** was synthesized following the general procedure for CuAAC and using azido-precursor **153** (200 μmol). The crude product was purified by silica gel column chromatography using a $\text{CH}_2\text{Cl}_2/\text{MeOH}$ gradient (0% to 20% MeOH) to yield a white solid (131 mg).

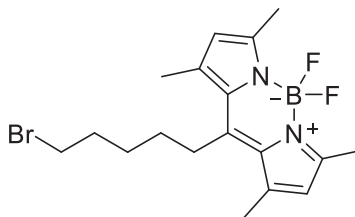
Yield 65%; mp: 135-141 $^\circ\text{C}$; ^1H NMR (600 MHz, $\text{DMSO}-d_6$) δ 0.94 (s, 9H), 1.19 – 1.28 (m, 2H), 1.38 (d, $J = 7.0$ Hz, 3H), 1.43 – 1.55 (m, 2H), 1.63 – 1.69 (m, 1H), 1.74 – 1.81 (m, 5H), 1.93 (quint, $J = 7.4$ Hz, 4H), 2.07 – 2.13 (m, 1H), 2.19 – 2.25 (m, 1H), 2.27 – 2.34 (m, 1H), 2.46 (s, 3H), 2.59 (t, $J = 7.4$ Hz, 4H), 2.78 (t, $J = 7.4$ Hz, 4H), 3.39 (dd, $J = 10.1, 5.2$ Hz, 1H), 3.90 (dd, $J = 10.3, 5.6$ Hz, 1H), 4.17 – 4.23 (m, 1H), 4.26 (t, $J = 7.2$ Hz, 2H), 4.33 (dd, $J = 8.7, 6.0$ Hz, 1H), 4.44 (d, $J = 8.7$ Hz, 1H), 4.89 – 4.96 (m, 1H), 5.31 (d, $J = 6.5$ Hz, 1H), 6.07 (s, 1H), 6.93 (s, 1H), 7.35 – 7.41 (m, 2H), 7.41 – 7.47 (m, 2H), 7.69 (s, 1H), 7.73 (s, 1H), 7.79 (d, $J = 8.8$ Hz, 1H), 7.89 (s, 1H), 8.09 (s, 1H), 8.29 (d, $J = 7.7$ Hz, 1H), 8.98 (s, 1H), 10.85 (s, 1H); ^{13}C NMR (151 MHz, $\text{DMSO}-d_6$) δ 15.9, 22.2, 24.7, 25.0, 25.5, 26.4, 29.4, 30.1, 30.6, 32.4, 34.5, 34.6, 36.8, 47.8, 49.2, 55.5, 56.7, 58.5, 69.0, 69.5, 117.9, 121.1, 126.4, 127.3, 128.8, 129.8, 131.1, 131.9, 137.2, 140.4, 143.0, 144.2, 147.8, 149.9, 151.5, 153.9, 169.9, 171.1, 172.2; **LC-MS** (ESI) (90% H_2O to 100% MeCN in 10 min, then 100% MeCN to 15 min, DAD 220-600 nm), $t_{\text{R}} = 5.53$ min, 97% purity, m/z $[\text{M} + \text{H}]^+$ calcd for $\text{C}_{50}\text{H}_{63}\text{N}_9\text{O}_8\text{S}_3$ 1014.40, found 1014.7; **HRMS** (ESI) m/z $[\text{M} + \text{H}]^+$ calcd for $\text{C}_{50}\text{H}_{63}\text{N}_9\text{O}_8\text{S}_3$ 1014.4034, found 1014.4027.

(2*S*,4*S*)-1-((2*S*)-2-(8-(4-(1-(5-(*N*-((1,2,3,5,6,7-Hexahydro-*s*-indacen-4-yl)carbamoyl)sulfamoyl)thiophen-3-yl)-1-hydroxyethyl)-1*H*-1,2,3-triazol-1-yl)octanamido)-3,3-dimethylbutanoyl)-4-hydroxy-*N*-((*S*)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)pyrrolidine-2-carboxamide (156)



Compound **156** was synthesized following the general procedure for CuAAC and using azido-precursor **154** (200 μmol). The crude product was purified by silica gel column chromatography using a $\text{CH}_2\text{Cl}_2/\text{MeOH}$ gradient (0% to 20% MeOH) to yield a white solid (118 mg).

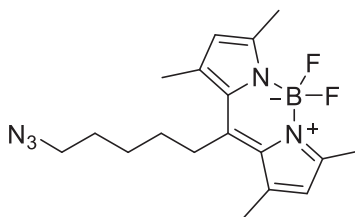
Yield 57%; mp: 133-139 $^\circ\text{C}$; ^1H NMR (600 MHz, $\text{DMSO}-d_6$) δ 0.95 (s, 9H), 1.17 – 1.24 (m, 6H), 1.38 (d, $J = 7.0$ Hz, 3H), 1.40 – 1.51 (m, 2H), 1.63 – 1.69 (m, 1H), 1.73 – 1.81 (m, 5H), 1.93 (quint, $J = 7.4$ Hz, 4H), 2.06 – 2.12 (m, 1H), 2.19 – 2.25 (m, 1H), 2.27 – 2.33 (m, 1H), 2.46 (s, 3H), 2.58 (t, $J = 7.4$ Hz, 4H), 2.78 (t, $J = 7.4$ Hz, 4H), 3.39 (dd, $J = 10.1, 5.2$ Hz, 1H), 3.90 (dd, $J = 10.2, 5.6$ Hz, 1H), 4.16 – 4.22 (m, 1H), 4.27 (t, $J = 7.2$ Hz, 2H), 4.33 (dd, $J = 8.7, 6.1$ Hz, 1H), 4.44 (d, $J = 8.8$ Hz, 1H), 4.89 – 4.96 (m, 1H), 5.31 (d, $J = 6.5$ Hz, 1H), 6.07 (s, 1H), 6.94 (s, 1H), 7.39 (d, $J = 8.0$ Hz, 2H), 7.43 (d, $J = 8.1$ Hz, 2H), 7.72 (d, $J = 1.4$ Hz, 1H), 7.74 (d, $J = 1.4$ Hz, 1H), 7.77 (d, $J = 8.8$ Hz, 1H), 7.88 (s, 1H), 8.11 (s, 1H), 8.28 (d, $J = 7.7$ Hz, 1H), 8.98 (s, 1H), 10.88 (s, 1H); ^{13}C NMR (151 MHz, $\text{DMSO}-d_6$) δ 15.9, 22.2, 25.0, 25.2, 25.8, 26.4, 28.0, 28.4, 29.6, 30.1, 30.6, 32.4, 34.6, 34.7, 36.8, 47.7, 49.2, 55.5, 56.6, 58.5, 69.0, 69.5, 118.0, 121.2, 126.4, 127.6, 128.6, 128.8, 129.8, 131.1, 132.1, 137.2, 140.0, 143.1, 144.2, 147.8, 148.9, 149.9, 151.4, 153.9, 169.9, 171.0, 172.3; LC-MS (ESI) (90% H_2O to 100% MeCN in 10 min, then 100% MeCN to 15 min, DAD 220-600 nm), $t_{\text{R}} = 5.96$ min, 97% purity, m/z $[\text{M} + \text{H}]^+$ calcd for $\text{C}_{52}\text{H}_{67}\text{N}_9\text{O}_8\text{S}_3$ 1042.43, found 1042.7; HRMS (ESI) m/z calcd for $\text{C}_{52}\text{H}_{67}\text{N}_9\text{O}_8\text{S}_3$ 1042.4347, found 1042.4347.

10-(5-Bromopentyl)-5,5-difluoro-1,3,7,9-tetramethyl-5H-4 λ ⁴,5 λ ⁴-dipyrrolo[1,2-*c*:2',1'-*f*][1,3,2]diazaborinine (157**)**^{302,349}

Compound **157** was prepared similar to a previously reported procedure.³⁰² 6-Bromohexanoyl chloride (391 mg, 1.83 mmol) was dissolved in dry CH₂Cl₂ (10 mL) and cooled to 0 °C under argon. 2,4-Dimethyl-1*H*-pyrrole (462 mg, 4.86 mmol) was added dropwise. The mixture was stirred for 30 min at 0 °C. The reaction mixture was allowed to warm to room temperature and was stirred for additional 30 min. The mixture was cooled to 0 °C and TEA (511 mg, 5.05 mmol) was added dropwise. The mixture was allowed to warm to room temperature and was stirred for additional 10 min. Boron trifluoride diethyl etherate (1.15 g, 8.10 mmol) was added dropwise. The reaction mixture was stirred for 14 h at room temperature under argon. The mixture was diluted with CH₂Cl₂ (30 mL) and the organic layer was washed with water (3 × 40 mL). The organic layer was dried over Na₂SO₄, filtered and concentrated *in vacuo*. The crude product was purified by silica gel column chromatography using petroleum ether/CH₂Cl₂ (1+1) as eluent to yield a red solid (183 mg).

Yield 25%; mp: 116-118 °C, lit.³⁴⁹ mp: 134-136 °C; ¹H NMR (500 MHz, DMSO-*d*₆) δ 1.57 – 1.63 (m, 4H), 1.84 – 1.93 (m, 2H, CH₂), 2.40 (s, 6H), 2.42 (s, 6H, CH₃), 2.92 – 2.99 (m, 2H, CH₂), 3.57 (t, *J* = 6.6 Hz, 2H, CH₂Br), 6.23 (s, 2H, CH_{arom.}); ¹³C NMR (126 MHz, DMSO-*d*₆) δ 14.0, 15.8 (CH₃), 27.6, 28.0, 30.3, 31.6, 34.9 (CH₂), 121.6, 130.7, 140.8, 146.5, 153.0 (C_{arom.}); LC-MS (ESI) (90% H₂O to 100% MeCN in 10 min, then 100% MeCN to 15 min, DAD 220-600 nm), *t*_R = 9.45 min, 91% purity, *m/z* [M - H]⁺ calcd for C₁₈H₂₄BBBrF₂N₂ 395.11, found 395.1.

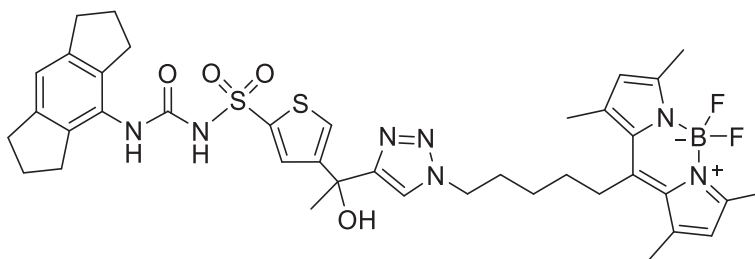
10-(5-Azidopentyl)-5,5-difluoro-1,3,7,9-tetramethyl-5H-4 λ^4 ,5 λ^4 -dipyrrolo[1,2-*c*:2',1'-*f*][1,3,2]diazaborinine (158**)**³⁰²



Compound **158** was prepared similar to a previously reported procedure.³⁰² Compound **157** (159 mg, 400 μ mol) was dissolved in dry DMF (5 mL). NaN₃ (156 mg, 2.40 mmol) was added and the reaction mixture was stirred at 50 °C for 18 h. The mixture was diluted with EtOAc (50 mL). The organic layer was washed saturated NH₄Cl solution (3 $\times$ 50 mL). The organic layer was dried over Na₂SO₄, filtered and concentrated *in vacuo* to yield a red solid (132 mg).

Yield 92%; mp: 80-82 °C; ¹H NMR (600 MHz, DMSO-*d*₆) δ 1.51 – 1.55 (m, 2H), 1.56 – 1.64 (m, 4H, CH₂), 2.40 (s, 6H), 2.42 (s, 6H, CH₃), 2.91 – 2.98 (m, 2H, CH₂), 3.36 (t, *J* = 6.8 Hz, 2H, CH₂N₃), 6.23 (s, 2H, CH_{arom.}); ¹³C NMR (151 MHz, DMSO-*d*₆) δ 14.0, 15.8 (CH₃), 26.8, 27.7, 27.9, 30.8 (CH₂), 50.5 (CH₂N₃), 121.7, 130.7, 140.8, 146.6, 153.0 (C_{arom.}); LC-MS (ESI) (90% H₂O to 100% MeCN in 10 min, then 100% MeCN to 15 min, DAD 220-600 nm), *t*_R = 9.24 min, 94% purity, *m/z* [M - H]⁻ calcd for C₁₈H₂₄BF₂N₅ 358.20, found 358.2.

4-(1-(1-(5-(5,5-Difluoro-1,3,7,9-tetramethyl-5H-4 λ^4 ,5 λ^4 -dipyrrolo[1,2-*c*:2',1'-*f*][1,3,2]diazaborinin-10-yl)pentyl)-1H-1,2,3-triazol-4-yl)-1-hydroxyethyl)-N-((1,2,3,5,6,7-hexahydro-*s*-indacen-4-yl)carbamoyl)thiophene-2-sulfonamide (159**)**

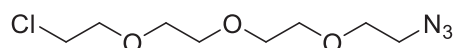


Compound **159** was synthesized following the general procedure for CuAAC and using azido-precursor **158** (200 μ mol). The crude product was purified by silica gel flash column chromatography using a CH₂Cl₂/MeOH gradient (0% to 20% MeOH) to yield an orange solid (37 mg).

Yield 23%; mp: 147-153 °C; ¹H NMR (600 MHz, DMSO-*d*₆) δ 1.37 – 1.43 (m, 2H), 1.53 – 1.59 (m, 2H, CH₂), 1.79 (s, 3H, CH₃CO), 1.84 – 1.89 (m, 2H, CH₂), 1.92 (quint, *J* = 7.5 Hz,

4H, CH₂ indacene), 2.36 (s, 6H), 2.40 (s, 6H, CH₃), 2.56 (t, $J = 7.4$ Hz, 4H, CH₂ indacene), 2.77 (t, $J = 7.4$ Hz, 4H, CH₂ indacene), 2.84 – 2.90 (m, 2H, CH₂), 4.32 (t, $J = 7.1$ Hz, 2H, CH₂), 6.08 (s, 1H, OH), 6.22 (s, 2H, CH_{arom.}), 6.92 (s, 1H, CH_{arom.} indacene), 7.72 (d, $J = 1.6$ Hz, 1H, CH_{arom.} thiophene), 7.73 (d, $J = 1.6$ Hz, 1H, CH_{arom.} thiophene), 7.89 (s, 1H, CH_{arom.} triazole), 8.10 (s, 1H, NH), 10.83 (s, 1H, SO₂NH); ¹³C NMR (151 MHz, DMSO-*d*₆) δ 14.0, 15.8, 25.0, 26.4, 27.6, 29.2, 30.1, 30.5, 30.6, 32.4, 48.9, 69.5, 118.0, 121.2, 121.6, 127.6, 128.5, 130.7, 132.2, 137.2, 140.0, 140.8, 143.1, 146.5, 148.9, 150.0, 153.0, 154.0; LC-MS (ESI) (90% H₂O to 100% MeOH in 10 min, then 100% MeOH to 15 min, DAD 220-600 nm), $t_R = 10.02$ min, 95% purity, m/z [M - H]⁻ calcd for C₃₉H₄₆BF₂N₇O₄S₂ 788.30, found 788.6; HRMS (ESI) m/z [M + H]⁺ calcd for C₃₉H₄₆BF₂N₇O₄S₂ 790.3193, found 790.3178.

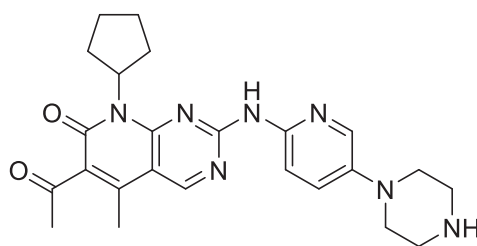
1-Azido-2-(2-(2-(2-chloroethoxy)ethoxy)ethoxy)ethane (**160**)³⁰⁷



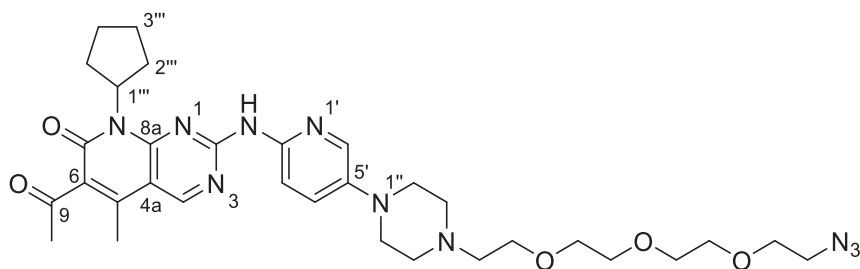
Compound **160** was prepared similar to a previously reported procedure.³⁰⁷ 1-Chloro-2-(2-(2-(2-chloroethoxy)ethoxy)ethoxy)ethane (994 mg, 4.30 mmol) was dissolved in dry DMF (10 mL). Sodium azide (293 mg, 4.50 mmol) was added. The mixture was stirred at 70 °C for 18 h. The mixture was carefully concentrated. The residue was taken up in H₂O (100 mL). The aqueous layer was carefully extracted with CH₂Cl₂ (3 × 75 mL). The combined organic layers were washed with H₂O (50 mL) and brine (50 mL), dried over Na₂SO₄, filtered and concentrated *in vacuo* to yield a colorless liquid (998 mg).

Yield 98%; ¹H NMR (600 MHz, CDCl₃-*d*) δ 3.38 (t, $J = 5.0$ Hz, 2H), 3.62 (t, $J = 5.9$ Hz, 2H), 3.64 – 3.69 (m, 10H), 3.75 (t, $J = 5.9$ Hz, 2H, CH₂); ¹³C NMR (151 MHz, CDCl₃-*d*) δ 42.8 (CH₂Cl), 50.8 (CH₂N₃), 70.1, 70.1, 70.8, 70.8, 71.4 (OCH₂), one signal is missing due to overlapping signals; LC-MS (ESI) (90% H₂O to 100% MeCN in 10 min, then 100% MeCN to 15 min), $t_R = 4.81$ min, m/z [M + NH₄]⁺ calcd for C₈H₁₆ClN₃O₃ 255.12, found 255.1.

Palbociclib (**161**)

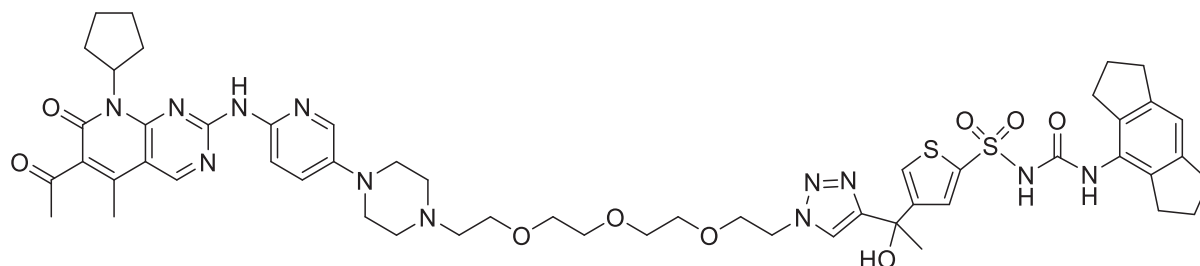


Compound **161** was purchased from Activate Scientific (Prien, Germany).



Yield 51%; **¹H NMR** (500 MHz, DMSO-*d*₆) δ 1.53 – 1.62 (m, 2H), 1.73 – 1.80 (m, 2H), 1.84 – 1.92 (m, 2H), 2.20 – 2.28 (m, 2H, 2'''-H, 3'''-H, 4'''-H, 5'''-H), 2.31 (s, 3H), 2.42 (s, 3H, CH₃), 2.52 – 2.56 (m, 2H), 2.57 – 2.63 (m, 4H), 3.11 – 3.18 (m, 4H), 3.36 – 3.42 (m, 2H), 3.51 – 3.58 (m, 10H), 3.59 – 3.63 (m, 2H, CH₂, 2''-H, 3''-H, 5''-H, 6''-H), 5.82 (quint, *J* = 8.9 Hz, 1H, 1'''-H), 7.45 (dd, *J* = 9.0, 3.0 Hz, 1H, 4'-H), 7.84 (d, *J* = 9.0 Hz, 1H, 3'-H), 8.04 (d, *J* = 3.0 Hz, 1H, 6'-H), 8.95 (s, 1H, 4-H), 10.06 (s, 1H, NH); **¹³C NMR** (126 MHz, DMSO-*d*₆) δ 13.5 (CH₃), 25.0 (C-3'''), 27.5 (C-2'''), 31.2 (C-10), 48.2, 50.0, 52.8, 52.9, 57.1 (C-2'', C-3'', C-5'', C-6'', CH₂N, CH₂N₃), 68.3, 69.2, 69.6, 69.7, 69.8, 70.5 (OCH₂), 106.5 (C-4a), 115.1 (C-3'), 124.5 (C-4'), 129.2 (C-6), 135.3 (C-6'), 142.0 (C-5'), 143.4, 144.2 (C-8a, C-2'), 154.7 (C-5), 158.1, 158.5 (C-4, C-2), 160.7 (C-7), 202.3 (C-9); **LC-MS** (ESI) (90% H₂O to 100% MeCN in 10 min, then 100% MeCN to 15 min, DAD 220-600 nm), *t*_R = 7.09 min, 97% purity, *m/z* [M + H]⁺ calcd for C₃₂H₄₄N₁₀O₅ 649.36, found 649.6.

4-(1-(1-(2-(2-(2-(2-(4-(6-((6-Acetyl-8-cyclopentyl-5-methyl-7-oxo-7,8-dihydropyrido[2,3-*d*]pyrimidin-2-yl)amino)pyridin-3-yl)piperazin-1-yl)ethoxy)ethoxy)ethoxy)ethyl)-1*H*-1,2,3-triazol-4-yl)-1-hydroxyethyl)-*N*-((1,2,3,5,6,7-hexahydro-*s*-indacen-4-yl)carbamoyl)thiophene-2-sulfonamide (163)



Compound **163** was synthesized following the general procedure for CuAAC and using azido-precursor **162** (150 μ mol). The crude product was purified by silica gel flash column chromatography using a $\text{CH}_2\text{Cl}_2/\text{MeOH}$ gradient (0% to 20% MeOH) to yield a yellow solid (93 mg).

Yield 57%; mp: 134-140 $^\circ\text{C}$; ^1H NMR (600 MHz, $\text{DMSO}-d_6$) δ 1.54 – 1.63 (m, 2H), 1.75 – 1.82 (m, 5H), 1.85 – 1.93 (m, 6H), 2.21 – 2.28 (m, 2H), 2.31 (s, 3H), 2.42 (s, 3H), 2.62 (t, J = 7.5 Hz, 4H), 2.65 – 2.70 (m, 2H), 2.70 – 2.78 (m, 8H), 3.14 – 3.20 (m, 4H), 3.47 – 3.53 (m, 8H), 3.56 (t, J = 5.7 Hz, 2H), 3.79 (t, J = 5.2 Hz, 2H), 4.47 (t, J = 5.1 Hz, 2H), 5.77 – 5.87 (m, 1H), 5.97 (s, 1H), 6.84 (s, 1H), 7.46 (s, 2H), 7.55 (s, 1H), 7.77 – 7.97 (m, 3H), 8.04 (s, 1H), 8.95 (s, 1H), 10.08 (s, 1H), one signal (1H, SO_2NH) is missing due to proton exchange; ^{13}C NMR (151 MHz, $\text{DMSO}-d_6$) δ 13.9, 25.0, 25.1, 27.5, 30.3, 30.9, 31.3, 32.5, 47.6, 49.3, 52.5, 52.9, 56.7, 67.5, 68.7, 69.5, 69.6, 69.6, 106.6, 115.0, 116.8, 121.6, 124.7, 129.2, 130.5, 135.4, 136.9, 142.0, 142.6, 143.1, 144.4, 149.3, 154.2, 154.7, 158.2, 158.5, 160.7, 202.4; LC-MS (ESI) (90% H_2O to 100% MeCN in 10 min, then 100% MeCN to 15 min, DAD 220-600 nm), t_R = 6.41 min, 97% purity, m/z $[\text{M} + \text{H}]^+$ calcd for $\text{C}_{53}\text{H}_{66}\text{N}_{12}\text{O}_9\text{S}_2$ 1079.46, found 1079.7; HRMS (ESI) m/z $[\text{M} + \text{H}]^+$ calcd for $\text{C}_{53}\text{H}_{66}\text{N}_{12}\text{O}_9\text{S}_2$ 1079.4590, found 1079.4586

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Appendix

This section contains the full-length publications of the chapters 2, 3 and 4, including the supporting information. Furthermore, this appendix contains the NMR spectra of final compounds reported in chapter 5. Please note that the copyright of the publications belongs to the publishers of the respective journals, as indicated by the copyright statements denoted before each paper.

Appendix I. Publication I: Development of the first non-hydroxamate selective HDAC6 degraders

The following pages include the article “Development of the first non-hydroxamate selective HDAC6 degraders” as it was published in Chemical Communications by the Royal Society of Chemistry.

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Development of the first non-hydroxamate selective HDAC6 degraders†

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The targeted degradation of histone deacetylase 6 (HDAC6) by heterobifunctional degraders constitutes a promising approach to treat HDAC6-driven diseases. Previous HDAC6 selective degraders utilised a hydroxamic acid as a zinc-binding group (ZBG) which features mutagenic and genotoxic potential. Here we report the development of a new class of selective HDAC6 degraders based on a difluoromethyl-1,3,4-oxadiazole warhead as ZBG.

Histone deacetylases (HDACs) are considered important epigenetic drug targets for the therapy of haematological and solid cancers.¹ Four HDAC inhibitors (HDACi; vorinostat, romidepsin, belinostat, and panobinostat) have received regulatory approval from the FDA for treating T-cell lymphoma and multiple myeloma. However, all approved HDACi do not possess selectivity for a specific HDAC isoform.¹ Due to their lack of isoform-selectivity, such unselective HDACi often cause suffering from serious adverse effects.¹ Thus, to optimise the risk-benefit profile of HDACi, there is urgent need to develop isoform-specific HDACi.

HDAC6 is overexpressed in various cancer types and modulates the activity of several non-histone proteins such as α -tubulin, cortactin, and Hsp90.² Since the knockout of HDAC6 in mice did not produce significant defects, HDAC6 inhibitors are considered to exhibit improved safety profiles compared to pan-HDACi.³ HDAC6 is structurally unique and comprises two active catalytic domains (CD1 and CD2) as well as a zinc finger functioning as a ubiquitin-binding domain (UBD). Classical HDAC6-selective inhibitors impede CD2 but do not interfere with enzymatic and non-enzymatic functions facilitated by CD1 or the UBD.¹ Hence, the chemical knockdown of HDAC6 may be superior to the sole inhibition of CD2.

Hijacking the ubiquitin-proteasome system (UPS) with proteolysis-targeting chimeras (PROTACs) is an emerging new therapeutic modality, which enables the targeted degradation of a protein of interest (POI). These heterobifunctional molecules consist of an E3 ligase ligand and a recognition motif for the POI connected by a suitable linker, thereby acting as proximity inducers.⁴ The formation of a POI: PROTAC: E3 ligase ternary complex initiates the polyubiquitination of the POI, leading to its proteasomal degradation.² In 2018, Schiedel *et al.*⁵ and Yang *et al.*⁶ reported the first Sirt2 and HDAC6 PROTACs, respectively. Several selective HDAC6 degraders have been disclosed in the past years, including compounds based on the HDAC6 inhibitor nexturastat A and the pan-HDACi crebinostat.^{6–10} All selective HDAC6 PROTACs reported so far contain a hydroxamate zinc-binding group (ZBG) which coordinates the zinc ion in the active site of HDAC6 CD2.^{6–10} Although hydroxamic acids have been successfully utilised as ZBGs in approved HDACi as well as in numerous late-stage clinical candidates, they may transform *via* Lossen rearrangements into mutagenic and highly reactive electrophilic species such as isocyanates susceptible to react with naturally occurring nucleophiles.¹¹ To avoid such mutagenic and genotoxic potential, new ZBGs are desirable for HDAC PROTAC development.¹¹ Herein, we report the first non-hydroxamate, selective HDAC6 PROTACs that contain difluoromethyl-1,3,4-oxadiazole warheads as ZBGs.

Selective HDAC6 inhibitors typically consist of a hydroxamate ZBG connected to a short benzyl or 4-aminophenyl linker and a bulky, rigid cap group that confers isoform selectivity. A few alternative ZBGs, for example, mercaptoacetamides, thiols, and trifluoromethyl ketones enabled potent HDAC6 inhibition but exhibited lower selectivity than hydroxamic acids.¹² In 2018, Yates¹³ disclosed a new type of highly potent HDAC6 selective inhibitors based on pyrimidine linkers and the 2-(difluoromethyl)-1,3,4-oxadiazole group as ZBG (for representative structures, see Fig. S1, ESI†). Inspired by this scaffold, we designed the *meta*- and *para*-connected HDAC6 ligands **I** and **II** (Fig. 1A) containing acetyl groups that mimic the PROTAC attachment points. Docking studies (Fig. S2, ESI†) to investigate their potential as POI ligands showed the acetyl group of the

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† Electronic supplementary information (ESI) available: Supplementary figures, schemes and tables, experimental procedures, ¹H NMR, ¹³C NMR and MS data, docking protocols. See DOI: <https://doi.org/10.1039/d2cc03712b>

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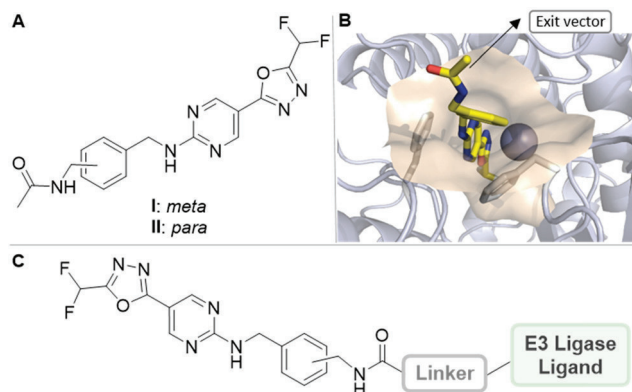


Fig. 1 (A) HDAC6 ligands **I** and **II** with possible *meta* and *para* attachment points. (B) Docking pose of ligand **I** in the CD2 of HDAC6 (PDB: 5EDU).¹⁴ The catalytic Zn^{2+} -ion is shown as gray sphere. (C) Designed *meta*- and *para*-connected HDAC6 PROTACs.

meta-substituted derivative **I** solvent-exposed (Fig. 1B), indicating an appropriate exit vector to assemble PROTACs. In turn, the predicted binding mode for the *para*-substituted analogue **II** suggests that the acetyl group binds in the proximity of Thr563 and Met567 (Fig. S3, ESI[†]). However, it seems sufficiently solvent-exposed. We thus decided to pursue both *meta*- and *para*-connected PROTACs and designed degraders that are capable of recruiting the well-studied E3 ubiquitin ligases cereblon (CRBN) and von Hippel-Lindau (VHL) (Fig. 1C).

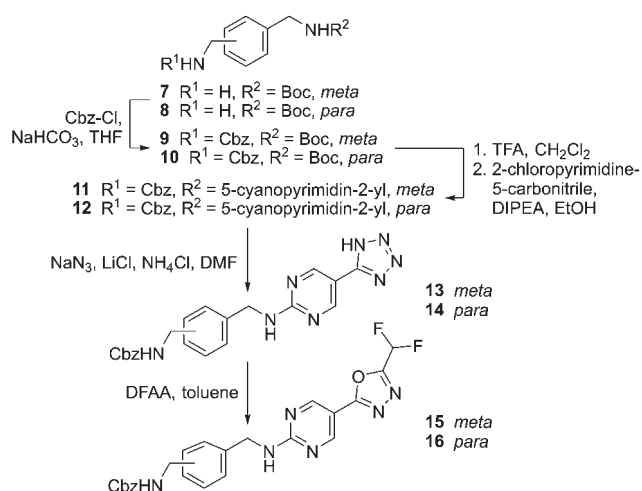
For the synthesis of the required HDAC6 ligands (Scheme 1) the mono-Boc-protected bis(aminomethyl)benzenes **7** and **8** were converted to the orthogonally protected building blocks **9** and **10**. After Boc-deprotection, the nucleophilic aromatic substitution of 2-chloropyrimidine-5-carbonitrile afforded the carbonitrile intermediates **11** and **12**, which were subjected to the reaction with sodium azide to provide **13** and **14**. For the preparation of the desired HDAC6 ligands **15** and **16**, the tetrazoles **13** and **14** were reacted with difluoroacetic anhydride

(DFAA) to generate the 2-(difluoromethyl)-1,3,4-oxadiazole group *via* a Huisgen 1,3,4-oxadiazole synthesis.¹⁵

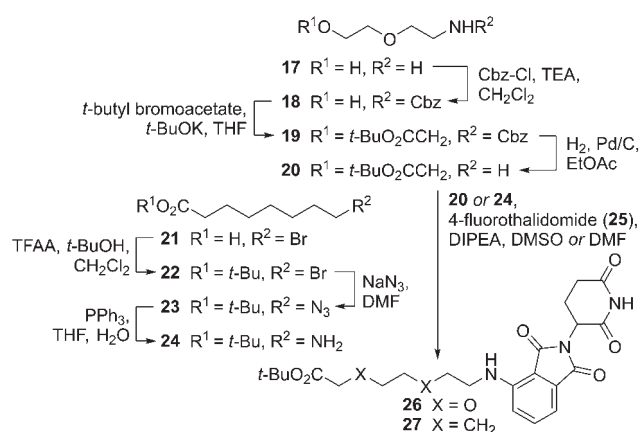
The cereblon-based PROTAC precursors were obtained *via* the synthetic route depicted in Scheme 2. First, a PEG-containing precursor (**26**) with pomalidomide as a cereblon binding unit was prepared. For this purpose, 2-(2-aminoethoxy)ethanol (**17**) was Cbz-protected to **18**, which was elongated through O-alkylation with *tert*-butyl bromoacetate. The resulting orthogonally protected linker **19** underwent catalytic hydrogenolysis to give the primary amine **20**. This was employed in a nucleophilic substitution reaction with 4-fluorothalidomide (**25**) to yield **26**.¹⁶ To evaluate the impact of compound polarity on degradation potency, we conceived the cereblon-based PROTAC precursor **27** with an alkylidene chain of equal length. Starting with 8-bromooctanoic acid (**21**), an esterification with *tert*-butanol and trifluoroacetic anhydride was performed,¹⁷ leading to **22**, which was transformed into the azide **23**, followed by a Staudinger reaction. The amine **24** was then conjugated with 4-fluorothalidomide (**25**) to obtain the second cereblon-based PROTAC precursor **27**.

The preparation of the VHL-based PROTAC precursor **34** (Scheme 3) started with the O-alkylation of **28** to give **29**, followed by hydrogenolytic deprotection. The resulting alcohol **30** was oxidised with (diacetoxyiodo)benzene (BAIB) and TEMPO to the carboxylic acid **31**. The Boc-protected VHL ligand **33** was synthesised in a convergent approach (Scheme S1, ESI[†]).¹⁸ Upon deprotection of **33**, it was linked to **31** in a uronium salt-mediated coupling to achieve the VHL-based PROTAC precursor **34**. The alkylidene precursor **35** was similarly obtained from unilaterally esterified undecanedioic acid **32**.

After hydrogenolytic deprotection of the HDAC6 ligands **15** and **16** and TFA-promoted deprotection of the PROTAC precursors **26**, **27**, **34** and **35**, respectively, the HDAC6 degraders **1–6** were finally assembled through amide coupling (Table 1). An overview of physicochemical properties (molecular weight, lipophilicity, plasma protein binding (PPB), number of rotatable bonds, polar surface area) to assess the drug-likeness of the degraders is provided in Table 1. Notably, introduction of oxygen atoms into the linker reduced both lipophilicity and plasma protein binding values.



Scheme 1 Synthesis of HDAC6 ligands with a 2-(difluoromethyl)-1,3,4-oxadiazole-based zinc-binding group.

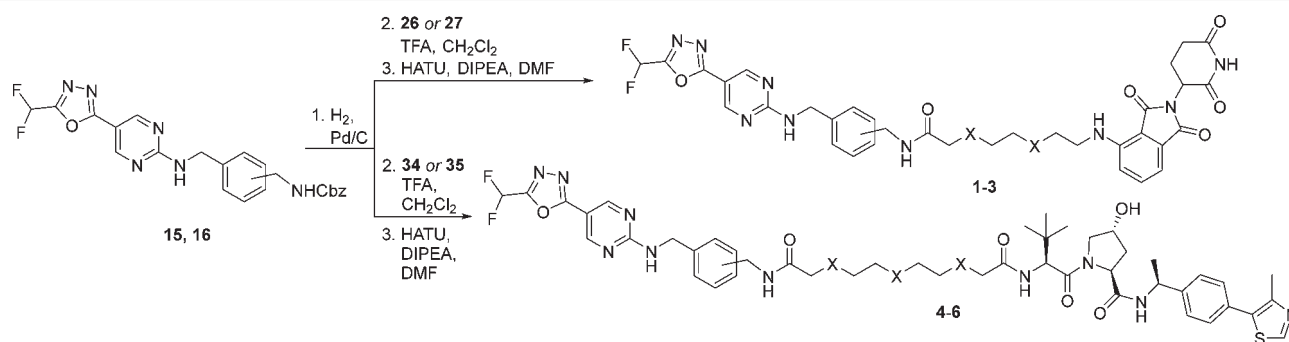


Scheme 2 Synthesis of precursors for cereblon-based PROTACs.

Scheme 3 Synthesis of precursors for VHL-based PROTACs.

In order to investigate whether the PROTACs **1–6** are capable of degrading HDAC6, we treated the multiple myeloma cell line MM.1S with 1 μ M of each degrader for 24 hours. HDAC6 degradation was subsequently determined by western blot analysis. As summarised in Table 1 and Fig. 2A, all compounds demonstrated a pronounced degradation of HDAC6. The most substantial degradation was achieved by compound **1** from the CRBN-recruiting series, whereas compound **4** displayed the

To confirm that degradation of HDAC6 is mediated by ternary complex formation, MM.1S cells were pretreated with the CRBN-ligand pomalidomide or the VHL-ligand VH298, followed by the addition of the PROTACs. As expected, degradation of HDAC6 induced by **1** (CRBN-recruiting) was blocked by pomalidomide, while VH298 only prevented the degradation



^a D_{max} , maximal degradation. ^b Experimental distribution coefficient at pH 7.4. ^c Experimentally determined percentage of compound bound to human serum albumin. ^d Topological polar surface area. ^e Number of rotatable bonds.

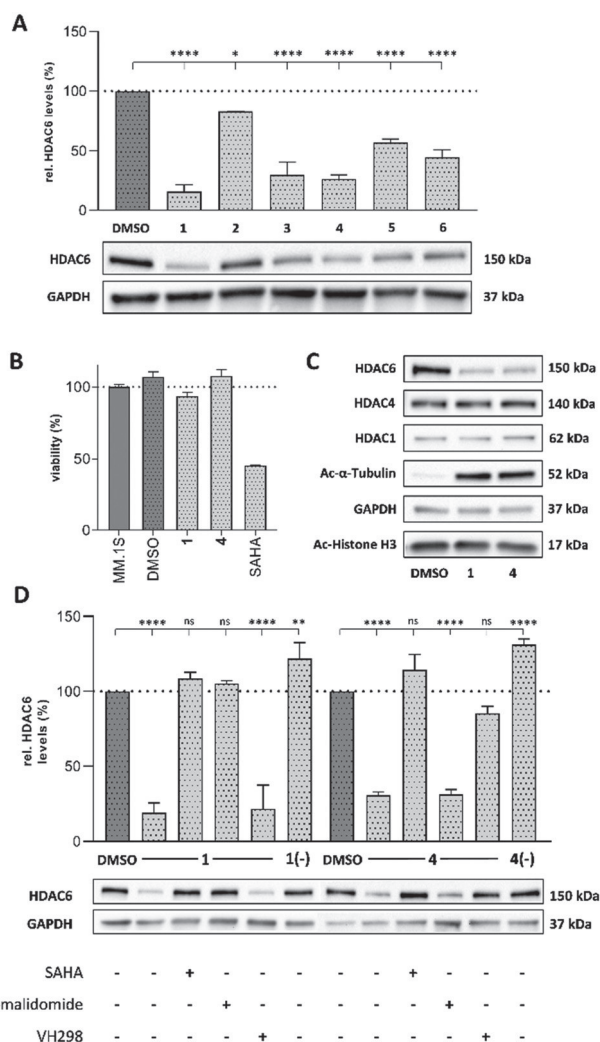


Fig. 2 Characterisation of non-hydroxamate HDAC6 PROTACs. (A) Western blot analysis of whole cell lysates of MM.1S cells after treatment with compounds **1–6** at 1 μ M for 24 h or with vehicle control (DMSO). (B) Investigation of potential cytotoxicity mediated by PROTACs **1** and **4** at a concentration of 1 μ M and after an incubation time of 72 h in MM.1S cells. SAHA (1 μ M) was used as cytotoxic control. (C) Protein levels of HDAC1, HDAC4, HDAC6, acetyl- α -tubulin, acetyl-histone H3 and GAPDH were quantified by western blot analysis after an incubation of 24 h with respective PROTACs **1** and **4** (1 μ M) in comparison to vehicle control (DMSO). (D) MM.1S cells were pre-treated with pomalidomide (10 μ M), VH298 (10 μ M), SAHA (1 μ M) or vehicle control (DMSO) for 30 min and then treated for 24 h with 1 μ M of **1** and **4**, respectively. Statistical analysis was performed by using one-way ANOVA followed by Dunnett's test. Statistical significance was indicated with asterisks (* = p < 0.05; ** = p < 0.01; *** = p < 0.001; **** = p < 0.0001).

activity of **4** (VHL-recruiting). Similarly, pretreatment with vorinostat (SAHA) rescued HDAC6 from degradation (Fig. 2D). To provide further evidence that HDAC6 degradation relied on ternary complex formation, we synthesised the non-degrading controls **1(-)** and **4(-)** by methylation of the glutarimide or by inversion of the stereochemistry at the hydroxyproline to

abolish the binding to the respective E3 ligase (see ESI† for structures and synthetic details). As illustrated in Fig. 2D, both control compounds showed no reduction in HDAC6 levels. Finally, rescue experiments with the NEDD8-activating enzyme inhibitor MLN4924 demonstrated that the degradation of HDAC6 occurs *via* the UPS (Fig. S6, ESI†).

In summary, we have designed, synthesised and evaluated the first non-hydroxamate HDAC6 degraders based on a difluoromethyl-1,3,4-oxadiazole warhead as ZBG. Western blot analysis demonstrated that the PROTACs **1** (CRBN-recruiting) and **4** (VHL-recruiting) are capable of degrading HDAC6 in a potent and selective manner. Considering the involvement of HDAC6 in various pathological conditions, the selective HDAC6 degraders reported in this work may be useful pharmacological tools to dissect the function of HDAC6 in cancer and non-oncological diseases.

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Conflicts of interest

There are no conflicts to declare.

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Supplementary Information

Development of the first non-hydroxamate selective HDAC6 degraders

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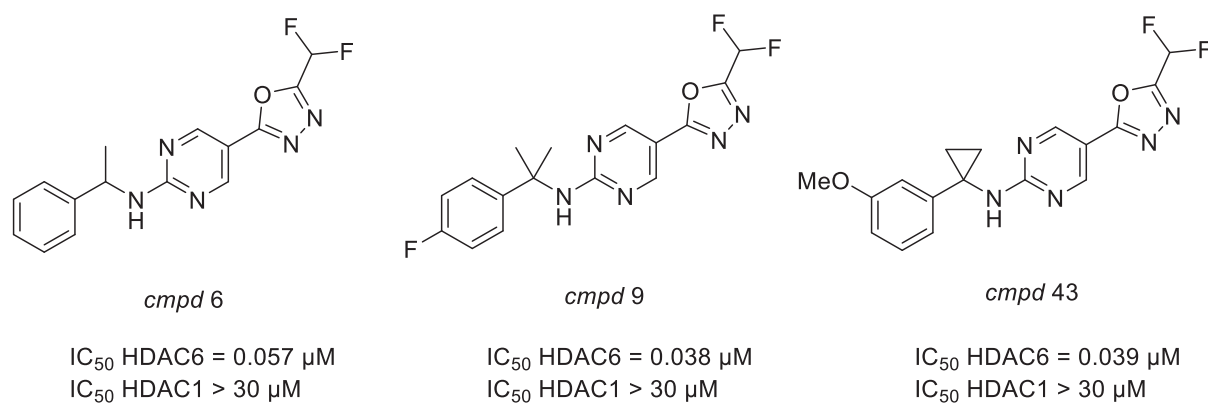


Figure S1. Selective HDAC6 inhibitors utilizing a 2-(difluoromethyl)-1,3,4-oxadiazole zinc-binding group. Structures and inhibition data taken from patent literature.¹

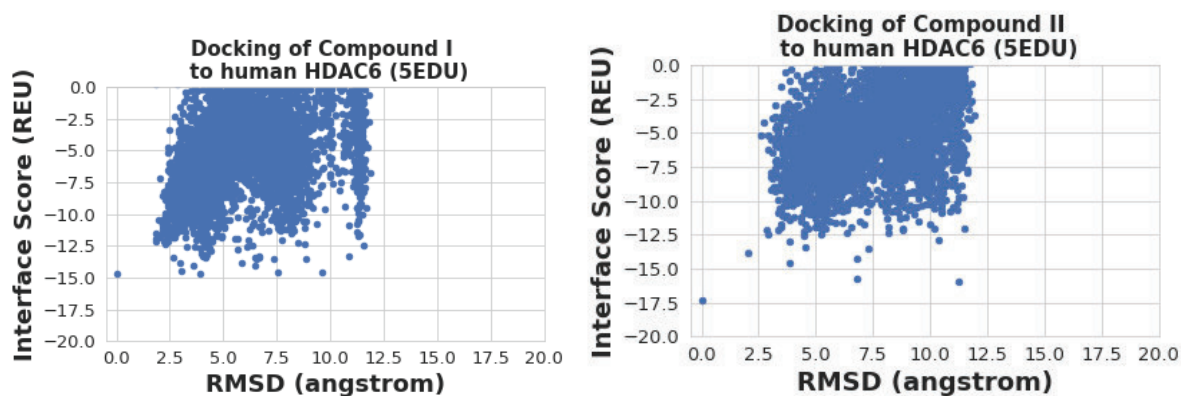


Figure S2. Ligand RMSD vs interface score plots for compound **I** and compound **II**. It was plotted against the models with the best scoring from their respective runs.

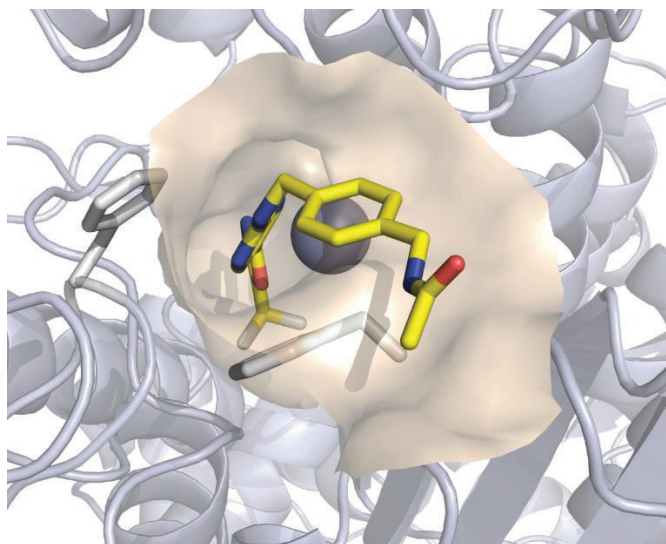


Figure S3. Docking pose of ligand **II** in the CD2 of HDAC6 (PDB: 5EDU).² The catalytic Zn²⁺-ion is shown as gray sphere.

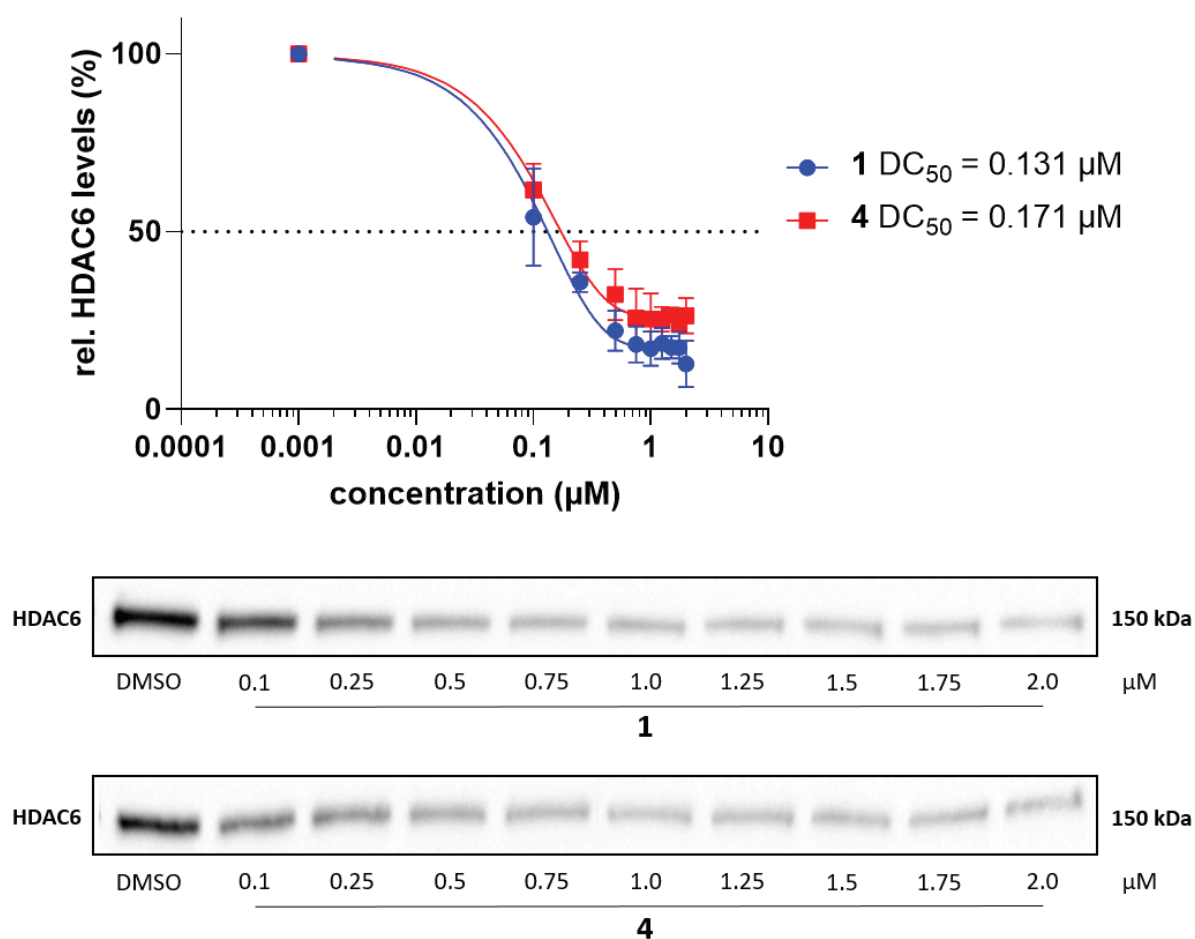


Figure S4. Determination of the half-degrading concentrations (DC₅₀) for PROTACs **1** and **4** in MM.1S cells. Multiple myeloma cells were incubated with several PROTAC concentrations ranging from 0.1 up to 2.0 μM.

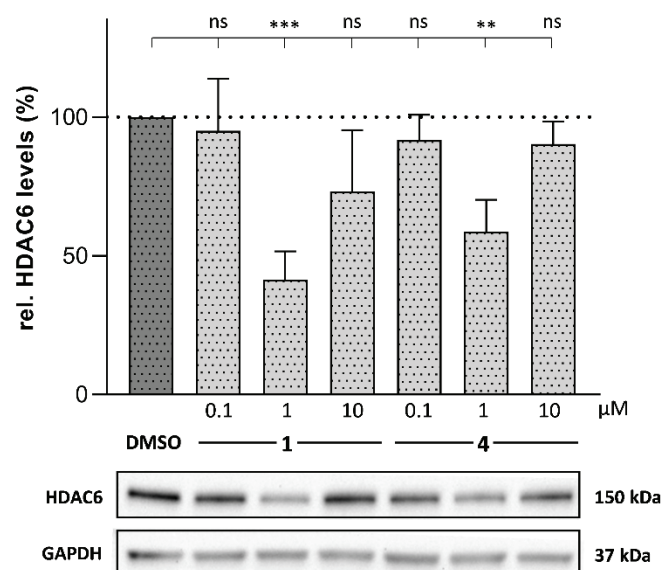


Figure S5. Investigation of concentration dependent degradation of HDAC6 induced by PROTACs **1** and **4**. Western blot analysis of MM.1S cells after treatment with compounds **1** and **4** at 0.1, 1 and 10 μM or with vehicle control (DMSO) for 6 h. At a concentration of 10 μM, a hook effect was observed for both compounds **1** and **4**. Statistical analysis was performed by using one-way ANOVA following Dunnett's test. Statistical significance was indicated with asterisks (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; **** = $p < 0.0001$).

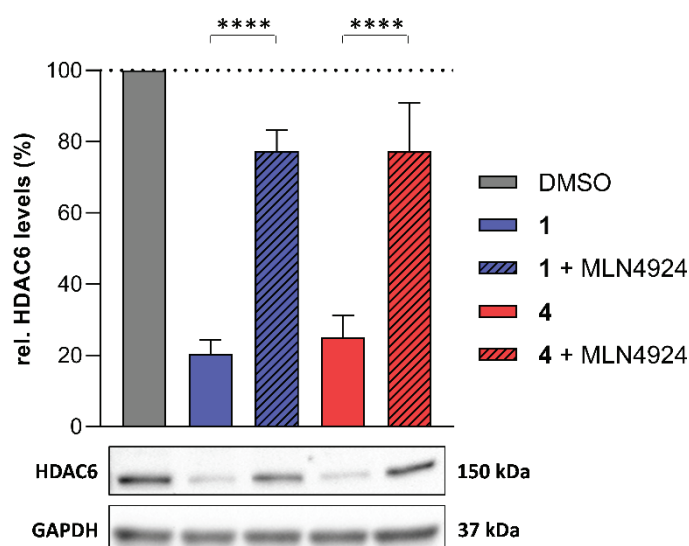
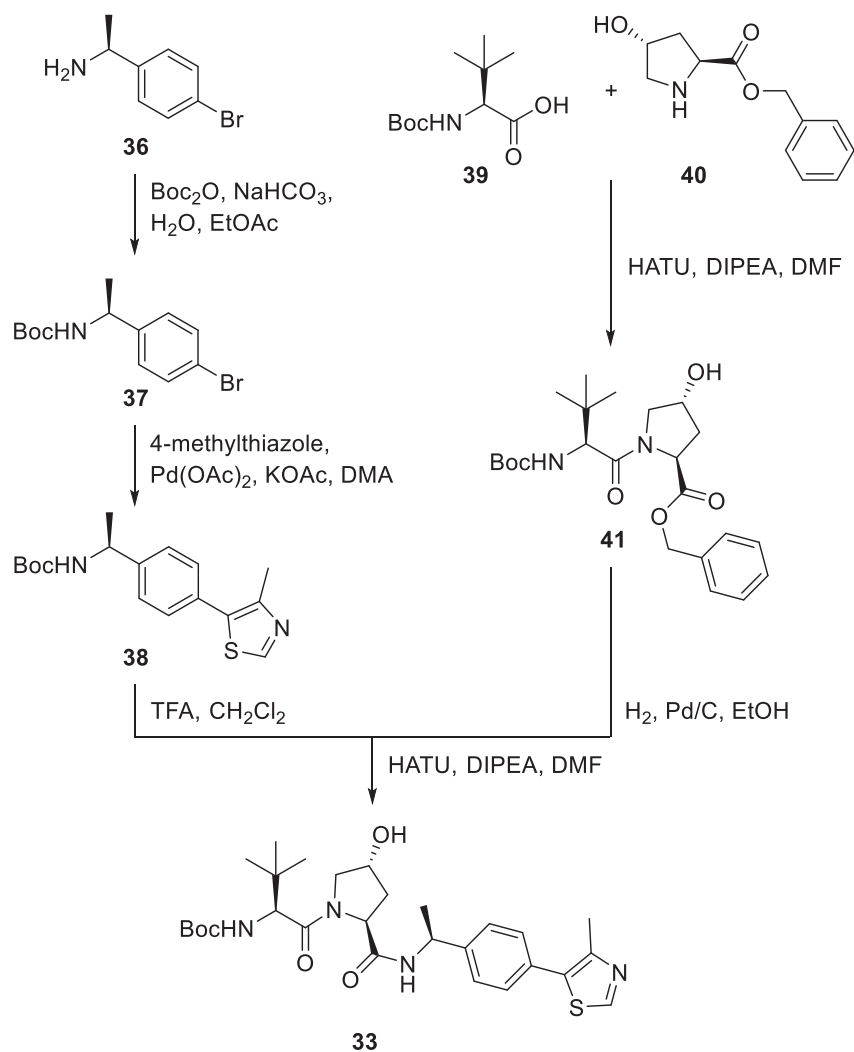


Figure S6. Elucidation of the involvement of the ubiquitin–proteasome system in the degradation of HDAC6 by western blot analysis. MM.1S cells were pre-treated with the NEDD8-activating enzyme inhibitor MLN4924 (1 μM) for 30 min and then treated for 24 h with 1 μM of **1** or **4**. DMSO was used as vehicle control. Statistical analysis was performed by using one-way ANOVA following Dunnett's test. Statistical significance was indicated with asterisks (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; **** = $p < 0.0001$).



Scheme S1. Synthesis of the VHL ligand **33**.

Table S1. Inhibitory activities (IC₅₀) of PROTACs **1-6** against HDAC1-4 and HDAC6.

cmpd	IC ₅₀ (μM)				
	HDAC1	HDAC2	HDAC3	HDAC4	HDAC6
1	> 30 ^a	> 30 ^a	> 30 ^a	> 30 ^a	0.643 ± 0.204
2	> 30 ^a	> 30 ^a	> 30 ^a	> 30 ^a	0.590 ± 0.133
3	> 30 ^a	> 30 ^a	> 30 ^a	> 30 ^a	1.86 ± 0.250
4	> 30 ^a	> 30 ^a	> 30 ^a	> 30 ^a	0.686 ± 0.113
5	> 30 ^a	> 30 ^a	> 30 ^a	> 30 ^a	1.68 ± 0.255
6	> 30 ^a	> 30 ^a	> 30 ^a	> 30 ^a	1.59 ± 0.123
vorinostat	0.102 ± 0.003	0.150 ± 0.022	0.077 ± 0.006	> 30 ^a	0.041 ± 0.004
TMP-269	n.d.	n.d.	n.d.	0.349 ± 0.059	n.d.

^a < 30% inhibition at 30 μM; n.d.: not determined.

2. Biological Experiments

2.1. Inhibition Assay for HDAC1-4 and HDAC6

In vitro inhibitory activities against HDAC1-3 and HDAC6 were measured using a previously published protocol.³ *In vitro* inhibitory activities against HDAC4 were measured using a previously published protocol with slight modifications.⁴ For test compounds and controls, serial dilutions of the respective DMSO stock solution in assay buffer (50 mM Tris-HCl, pH 8.0, 137 mM NaCl, 2.7 mM KCl, 1.0 mM MgCl₂·6H₂O, 0.1 mg/mL BSA) were prepared, and 5.0 µL of this serial dilution were transferred into OptiPlate-96 black microplates (PerkinElmer). In the case of HDAC1-3 and HDAC6, a volume of 35 µL of the fluorogenic substrate ZMAL (Z- Lys(Ac)-AMC,⁵ 21.43 µM in assay buffer) and 10 µL enzyme solution were added. In the case of HDAC4, 35 µL of the fluorogenic substrate Boc-Lys(Tfa)-AMC (Bachem, Catalog# 4060676, 42.86 µM in assay buffer) were added, followed by 10 µL of enzyme solution. Human recombinant HDAC1 (BPS Bioscience, Catalog# 50051), HDAC2 (BPS Bioscience, Catalog# 50052), HDAC3/NcoR2 (BPS Bioscience, Catalog# 50003), HDAC4 (BPS Bioscience, Catalog# 50004), or HDAC6 (BPS Bioscience, Catalog# 50006) was applied. The total assay volume of 50 µL (HDAC6 max. 1% DMSO; HDAC1-4 max. 5% DMSO) was incubated at 37 °C for 90 min. Subsequently, 50 µL of trypsin (HDAC1-3 and HDAC6: 0.4 mg/mL; HDAC4: 1.0 mg/mL) in trypsin buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl) was added, followed by additional 30 min of incubation at 37 °C. Fluorescence (excitation: 355 nm, emission: 460 nm) was measured using a FLUOstar OPTIMA microplate reader (BMG LABTECH). All compounds were tested at least twice in duplicates and the 50% inhibitory concentration (IC₅₀) was determined by plotting dose response curves and nonlinear regression with GraphPad Prism.

2.2. Cell Culture

The human multiple myeloma cell line MM.1S (CRL-2974) was obtained from ATCC (Manassas, VA, USA). MM.1S cells were cultivated in RPMI 1640 medium supplemented with 10% FBS, 100 IU/mL penicillin, 0.1 mg/mL streptomycin (PAN Biotech GmbH; Aidenbach, Germany) and 1 mM sodium pyruvate (ThermoFisher Scientific Inc.; Waltham, MA, USA) at 37 °C in a 5% CO₂ atmosphere. The semi-adherent cells were detached mechanically by using a cell scraper. The cell line was tested to exclude mycoplasma contamination every two weeks by qPCR.

2.3. Western Blot

MM.1S cells were seeded to cell culture flasks (75 cm²) and cultured for 96 h. Cells were then incubated for 24 h with the respective PROTACs at a concentration of 1 µM. In case of rescue experiments, cells were pre-incubated with the CRBN, VHL or HDAC6 ligands for 30 min before addition of the PROTACs. The next day, cell protein lysates were prepared from cells while incubating them at 4 °C on a shaker with cell extraction buffer (Life Technologies; Carlsbad, CA, USA) for 30 min. Lysed cells were afterwards centrifuged, the supernatant was collected and protein concentrations of the lysates

were quantified by a BCA protein assay kit (ThermoFisher Scientific Inc.). SDS-Page and western blots were conducted as described using stain-free gels.⁶ After proteins were transferred to PVDF membranes, western blots were incubated with blocking solution (milk powder-solution 5%) for 1 h at room temperature. Subsequently, mouse anti-HDAC1, rabbit anti-HDAC4, rabbit anti-HDAC6, rabbit anti-acetyl-histone H3, rabbit anti-acetyl- α -tubulin (Cell Signaling Technology; Frankfurt am Main, Germany) or mouse anti-GAPDH (GeneTex; Irvine, USA) antibody solutions were added to membranes, depending on the respective target. Next, HRP-conjugated secondary anti-rabbit (R&D systems; Minneapolis, MN, USA) and anti-mouse (Santa Cruz Biotechnology; Heidelberg, Germany) antibodies were used to quantify the proteins of interest by means of a chemiluminescence reaction. For visualisation and analysis of the western blot, we used the Clarity Western ECL substrate chemiluminescence kit, a ChemiDoc XRS+ imaging acquiring system and Image Lab software v.5.2.1 from BioRad Laboratories GmbH (Munich, Germany). For normalization, stainfree protein normalization and GAPDH as loading control were used.

2.4. Cell Viability Assay

To exclude possible cytotoxic effects of the PROTACs, cell viability of MM.1S cells was determined by using CellTiter-Glo[®] 2.0 luminescent cell viability assay (Promega; Madison, WI, USA). Cells were seeded in 96-well plates (25.000 tumour cells/well) and incubated with PROTACs for 72 h at a concentration of 1 μ M and 10 μ M, respectively. FDA-approved HDACi vorinostat (SAHA) was used as a cytotoxic positive control at the same concentrations as PROTACs, and DPBS as a non-cytotoxic negative control. After incubation, 100 μ L of CellTiter Glo 2.0 substrate were added to each well. In an ATP-dependent conversion of luciferin by a luciferase, conclusions can be drawn regarding the cytotoxic potential of the compounds. Luminescence readout is directly proportional to the number of viable cells.

2.5. Statistical Analysis

The results are shown as mean $\pm$ SD. Statistical analysis was performed by using one-way ANOVA following Dunnett's test. Statistical significance was indicated with asterisks (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; **** = $p < 0.0001$).

3. Molecular Docking

3.1. Procedure

The crystal structure of human HDAC6 (PDB: 5EDU)² was obtained from the Protein Data Bank (PDB, www.rcsb.org). Chain A, the maltose-binding periplasmic protein, and trichostatin A were deleted. All heteroatom records were removed, except for the metal ions (one zinc atom and two potassium atoms). The structure was optimized to the closest local energy minimum using RosettaRelax with coordinate constraints on the backbone and metal ion restraints.⁷ Ligand input files for compound **I** and **II** were created with ChemDraw. An initial 3D conformer with hydrogen atoms was constructed in Chem3D and energetically minimized using the MM2 force field, followed by the production of an ensemble of 1000 low-energy conformers with BCL:ConformerGenerator.⁸ One conformer was placed in the binding pocket of HDAC6. A constraint file was constructed to ensure binding of the amine of the linker to Ser453.⁹ Ligand docking was performed with RosettaLigand for an initial 5000 models. Those models were clustered according to their similarity in their binding mode. The depicted models are the best scoring models from their respective runs.¹⁰⁻¹² Rosetta version 3.12 was used. The executed commands used throughout the modeling process are provided by the authors on demand.

4. Physicochemical Experiments

4.1. Molecular Descriptor Calculations

Predicted values for the topological polar surface area (TPSA) and the number of rotatable bonds were calculated using the web service www.swissadme.ch provided by the Swiss Institute of Bioinformatics.

4.2. HPLC-based Determinations of $\log D_{7.4}$

The determination of the $\log D_{7.4}$ values was performed by a chromatographic method as described previously.^{13,14} The system was calibrated by plotting the retention times of six different drugs (atenolol, metoprolol, labetalol, diltiazem, triphenylene, permethrin) *versus* their literature known $\log D_{7.4}$ values to obtain a calibration line ($R^2 = 0.99$). Subsequently, the mean retention times of the analytes were taken to calculate their $\log D_{7.4}$ values with aid of the calibration line.

4.3. Plasma Protein Binding Studies

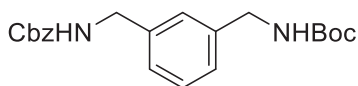
Plasma protein binding (PPB) was estimated by correlating the logarithmic retention times of the analytes on a CHIRALPAK HSA 50×3 mm, $5 \mu\text{m}$ column with the literature known percentage PPB values (converted into $\log K$ values) of the following drugs, warfarin, ketoprofen, budesonide, nizatidine, indomethacin, acetylsalicylic acid, carbamazepine, piroxicam, nicardipine, and cimetidine (for details, see Valko *et al.*¹⁵). Samples were dissolved in MeCN/DMSO 9:1 to achieve a final concentration of 0.5 mg/mL. The mobile phase A was 50 mM ammonium acetate adjusted to pH 7.4 with aqueous ammonia, while mobile phase B was *i*PrOH. The flow rate was set to 1.0 mL/min, the UV detector was set to 254 nm, and the column temperature was kept at 30 °C. After injecting 3 μL of the sample, a linear gradient from 100% A to 30% *i*PrOH in 5.4 min was applied. From 5.4 to 18 min, 30% *i*PrOH was kept, followed by switching back to 100% A in 1.0 min and a re-equilibration time of 6 min. With the aid of the calibration line ($R^2 = 0.96$), the $\log K$ values of new substances were calculated and converted to their %PPB values.

5. Chemical Experiments

5.1. General Information

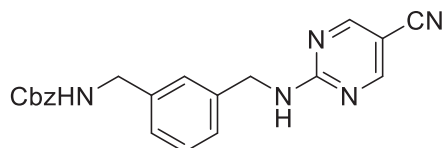
Chemicals were purchased from ABCR, Acros Organics, BLDpharm, Carl Roth, Fisher Scientific, Fluorochem, Sigma Aldrich, Tokyo Chemical Industry and VWR Chemicals. Thin layer chromatography was carried out with pre-coated silica gel (60 F₂₅₄) aluminum sheets from Merck. Detection was performed with UV light at 254 and 360 nm or with AgNO₃ or ninhydrin staining. Acros Organics silica gel 60 (70–230 mesh) was taken for preparative column chromatography. Preparative silica gel flash column chromatography was performed on an Interchim puriFlash XS520Plus with diode-array detection (DAD) from 200 to 400 nm. Uncorrected melting points were measured on a Büchi 510 oil bath apparatus or on a Büchi Melting Point M-565 apparatus. ESI-MS (LC-MS) analyses for compounds **1-6**, **18-48** and the chemical negative controls **1(-)** and **4(-)** respectively, were carried out on an API 2000 mass spectrometer coupled with an Agilent HPLC HP 1100 using an EC50/2 Nucleodur C18 Gravity 3 μ m column or on an Agilent Infinity Lab LC/MSD-system coupled with an Agilent HPLC 1260 Infinity II using an EC50/2 Nucleodur C18 Gravity 3 μ m column. The purity of synthesized compounds was determined by HPLC-DAD. HPLC measurements for compounds **15** and **16** were performed on a Thermo Fisher Scientific UltiMate™ 3000 UHPLC system with a Nucleodur 100-5 C18 column (250 $\times$ 4.6 mm, Macherey Nagel) with a flow rate of 1 mL/min and a temperature of 25 °C with an appropriate gradient. Detection was implemented by UV absorption measurement at a wavelength of λ = 254 nm. Bidest. H₂O (A) and MeCN (B) were used as eluents with an addition of 0.1% TFA for eluent A. Low resolution electrospray ionisation mass spectra (LRMS) were acquired with an Advion expression compact mass spectrometer coupled with an automated Advion TLC plate reader Plate Express. HR-ESI-MS spectra were recorded on a Bruker microOTOF-Q mass spectrometer coupled with a HPLC Dionex UltiMate 3000 or a LTQ Orbitrap XL. NMR spectra were recorded on a Bruker Avance DRX 500 (500 MHz ¹H NMR, 126 MHz ¹³C NMR) and a Bruker Avance III 600 (600 MHz ¹H NMR, 151 MHz ¹³C NMR). Chemical shifts are given in parts per million (ppm) referring to the signal center using the solvent peaks for reference, DMSO-*d*₆ (2.49/39.7).

5.2. Preparation of Compounds



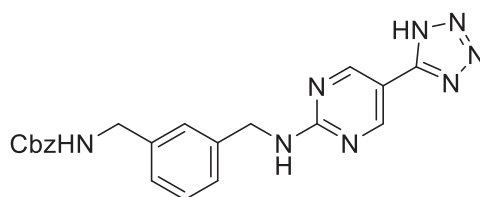
Benzyl (3-(((*tert*-butoxycarbonyl)amino)methyl)benzyl)carbamate (9). *tert*-Butyl (3-(aminomethyl)benzyl)carbamate (**7**, 803 mg, 3.40 mmol, 1.0 eq) was dissolved in THF (34 mL). NaHCO₃ (342 mg, 4.08 mmol, 1.2 eq) was added and the mixture was stirred at 0 °C. Subsequently, benzyl chloroformate (0.523 mL, 628 mg, 3.74 mmol, 1.1 eq) was added slowly and the reaction was allowed to stir for 12 h at room temperature. The mixture was quenched with water (60 mL) and extracted with EtOAc (3 × 60 mL). The combined organic layers were washed with brine (45 mL), dried over Na₂SO₄, filtered and concentrated under reduced pressure. The crude product was purified by silica gel column chromatography using cyclohexane/EtOAc (3+1), as eluent to yield **9** as a white solid (1.22 g, 3.31 mmol).

Yield 97%; mp. 99-102 °C; *R*_f = 0.34 (cyclohexane/EtOAc (3+1)); ¹H NMR (600 MHz, DMSO-*d*₆) δ 1.38 (s, 9H), 4.09 (d, *J* = 6.2 Hz, 2H), 4.18 (d, *J* = 6.2 Hz, 2H), 5.03 (s, 2H), 7.07 – 7.13 (m, 3H), 7.24 (t, *J* = 7.5 Hz, 1H), 7.28 – 7.33 (m, 2H), 7.36 (d, *J* = 6.4 Hz, 4H), 7.78 (t, *J* = 6.2 Hz, 1H); ¹³C NMR (126 MHz, DMSO-*d*₆) δ 28.4, 43.5, 44.0, 65.5, 77.9, 125.5, 125.7, 127.8, 127.9, 128.3, 128.5, 137.3, 139.8, 140.4, 155.9, 156.5; **LRMS (ESI)** *m/z* [M+H]⁺ calcd for C₂₁H₂₆N₂O₄ 371.2, found 371.1.



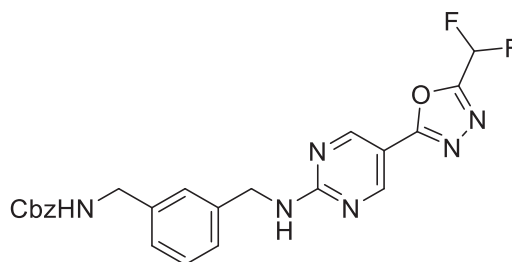
Benzyl (3-(((5-cyanopyrimidin-2-yl)amino)methyl)benzyl)carbamate (11). Benzyl (3-(((*tert*-butoxycarbonyl)amino)methyl)benzyl)carbamate (**9**, 1.22 g, 3.31 mmol, 1.0 eq) was dissolved in CH₂Cl₂ (26 mL), treated with TFA (7 mL) and stirred for 1 h at room temperature. After evaporation of the solvent, the resulting solid was dissolved in EtOH (11 mL), DIPEA (1.73 mL, 1.28 g, 9.93 mmol, 3.0 eq) and 2-chloropyrimidine-5-carbonitrile (924 mg, 6.62 mmol, 2.0 eq) were added and the reaction stirred at 80 °C for 24 h. The solvent was evaporated under reduced pressure, the residue was dissolved in EtOAc (200 mL), washed with brine (80 mL), dried over Na₂SO₄, filtered and concentrated under reduced pressure. The crude product was purified by silica gel column chromatography using cyclohexane/EtOAc (2+1) as eluent to afford **11** as a yellow solid (1.21 g, 3.26 mmol).

Yield 98%; mp. 122-128 °C; *R*_f = 0.26 (cyclohexane/EtOAc (2+1)); ¹H NMR (600 MHz, DMSO-*d*₆) δ 4.18 (d, *J* = 6.2 Hz, 2H), 4.54 (d, *J* = 6.4 Hz, 2H), 5.03 (s, 2H), 7.13 (d, *J* = 7.6 Hz, 1H), 7.16 (d, *J* = 7.6 Hz, 1H), 7.19 (d, *J* = 1.8 Hz, 1H), 7.26 (t, *J* = 7.6 Hz, 1H), 7.29 – 7.40 (m, 5H), 7.79 (t, *J* = 6.2 Hz, 1H), 8.66 – 8.71 (m, 2H), 8.82 (t, *J* = 6.4 Hz, 1H); ¹³C NMR (151 MHz, DMSO-*d*₆) δ 43.9, 44.1, 65.5, 95.6, 117.2, 125.7, 125.8, 125.9, 127.9, 127.9, 128.4, 128.5, 137.3, 139.1, 140.0, 156.5, 161.6, 161.9; **LRMS (ESI)** *m/z* [M+H]⁺ calcd for C₂₁H₁₉N₅O₂ 374.2, found 374.2.



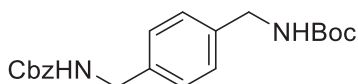
Benzyl (3-(((5-(1H-tetrazol-5-yl)pyrimidin-2-yl)amino)methyl)benzyl)carbamate (13). Benzyl (3-(((5-cyanopyrimidin-2-yl)amino)methyl)benzyl)carbamate (**11**, 205 mg, 0.550 mmol, 1.0 eq) was dissolved in DMF (2.2 mL). NaN₃ (71.5 mg, 1.1 mmol, 2.0 eq), NH₄Cl (38.5 mg, 0.720 mmol, 1.3 eq) and LiCl (11.8 mg, 0.280 mmol, 0.5 eq) were added and the reaction mixture was stirred at 100 °C for 18 h. After completion of the reaction, the mixture was quenched with ice water (8 mL) and acidified with 1 M HCl. The precipitated solid was filtered and washed with cold water to obtain the product **13** as a white solid (224 mg, 0.540 mmol).

Yield 98%; mp. 164-169 °C; ¹H NMR (500 MHz, DMSO-*d*₆) δ 4.18 (d, *J* = 6.2 Hz, 2H), 4.57 (d, *J* = 5.9 Hz, 2H), 5.02 (s, 2H), 7.12 (d, *J* = 7.5 Hz, 1H), 7.18 – 7.25 (m, 3H), 7.25 – 7.39 (m, 5H), 7.78 (t, *J* = 6.3 Hz, 1H), 8.43 (t, *J* = 6.5 Hz, 1H), 8.88 (s, 2H); ¹³C NMR (126 MHz, DMSO-*d*₆) δ 44.0, 44.2, 65.5, 108.0, 125.6, 125.7, 125.9, 127.8, 127.9, 128.4, 128.5, 137.3, 139.8, 140.0, 156.5, 156.7, 157.0, 162.8; **LRMS (ESI)** *m/z* [M+H]⁺ calcd for C₂₁H₂₀N₈O₂ 417.2, found 417.2.



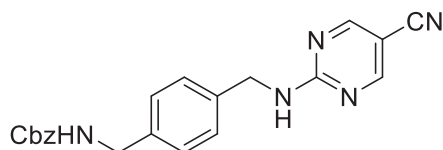
Benzyl (3-(((5-(5-(difluoromethyl)-1,3,4-oxadiazol-2-yl)pyrimidin-2-yl)amino)methyl)benzyl)carbamate (15). Benzyl (3-(((5-(1H-tetrazol-5-yl)pyrimidin-2-yl)amino)methyl)benzyl)carbamate (**13**, 454 mg, 1.09 mmol, 1.0 eq) was dissolved in toluene (10 mL). Difluoroacetic anhydride (DFAA, 0.403 mL, 569 mg, 3.27 mmol, 3.0 eq) was added and it was stirred at 70 °C for 18 h. The reaction mixture was concentrated under reduced pressure and purified by silica gel flash column chromatography using a CH₂Cl₂/MeOH gradient (0% to 5% MeOH) as eluent to yield **15** as a white solid (234 mg, 0.502 mmol).

Yield 46%; mp. 148-152 °C; ¹H NMR (600 MHz, DMSO-*d*₆) δ 4.17 (d, *J* = 6.2 Hz, 2H), 4.58 (d, *J* = 6.4 Hz, 2H), 5.01 (s, 2H), 7.12 (d, *J* = 7.5 Hz, 1H), 7.19 (d, *J* = 7.7 Hz, 1H), 7.21 – 7.39 (m, 7H), 7.51 (t, *J* = 51.4 Hz, 1H), 7.78 (t, *J* = 6.2 Hz, 1H), 8.71 (t, *J* = 6.4 Hz, 1H), 8.87 (s, 2H); ¹³C NMR (151 MHz, DMSO-*d*₆) δ 44.3, 44.5, 65.8, 106.8, 107.1 (t, *J* = 238.3 Hz), 126.1, 126.2, 128.2, 128.2, 128.8, 128.8, 137.6, 139.7, 140.3, 156.8, 157.5, 157.7, 158.0 (t, *J* = 28.9 Hz), 163.4, 163.6; ¹⁹F NMR (565 MHz, DMSO-*d*₆) δ -121.1, -121.2; **HRMS (ESI)** *m/z* [M+H]⁺ calcd for C₂₃H₂₀F₂N₆O₃ 467.1638, found 467.1610; **HPLC** (95% H₂O 5 min, then to 95% MeCN in 5 min, then 100% MeCN to 20 min, 254 nm), *t*_R = 16.32 min, 99% purity.



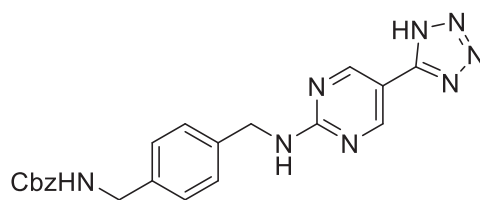
***tert*-Butyl (4-(((benzyloxy)carbonyl)amino)methyl)benzyl)carbamate (10).** 1-(*N*-Boc-aminomethyl)-4-(aminomethyl)benzene (**8**, 236 mg, 1.00 mmol, 1.0 eq.) was dissolved in THF (15 mL). NaHCO₃ (84.0 mg, 1.20 mmol, 1.2 eq) was added and the mixture was stirred at 0 °C. Subsequently, benzyl chloroformate (0.154 mL, 188 mg, 1.10 mmol, 1.1 eq) was added slowly and the reaction was allowed to stir for 12 h at room temperature. The mixture was quenched with water (20 mL) and extracted with EtOAc (3 × 20 mL). The combined organic layers were washed with brine (15 mL), dried over Na₂SO₄, filtered and concentrated under reduced pressure. The crude product was purified by silica gel column chromatography using cyclohexane/EtOAc (3+1), as eluent to yield **10** as a white solid (306 mg, 0.830 mmol).

Yield 83%; mp. 129-133 °C; *R*_f = 0.26 (cyclohexane/EtOAc (3+1)); ¹H NMR (600 MHz, DMSO-*d*₆) δ 1.38 (s, 9H), 4.08 (d, *J* = 6.2 Hz, 2H), 4.16 (d, *J* = 6.2 Hz, 2H), 5.03 (s, 2H), 7.15 – 7.19 (m, 5H), 7.29 – 7.37 (m, 5H), 7.76 (t, *J* = 6.2 Hz, 1H); ¹³C NMR (151 MHz, DMSO-*d*₆) δ 28.4, 43.3, 43.8, 65.5, 77.9, 127.0, 127.1, 127.9, 127.9, 128.5, 137.3, 138.2, 138.9, 155.9, 156.5; LRMS (ESI) *m/z* [M+H]⁺ calcd for C₂₁H₂₆N₂O₄ 371.2, found 371.1.



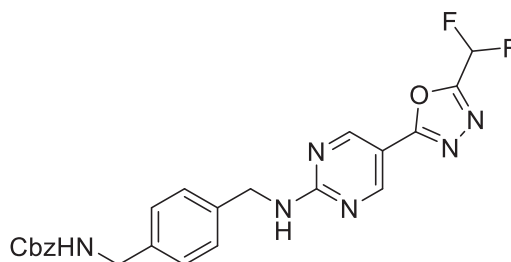
Benzyl (4-(((5-cyanopyrimidin-2-yl)amino)methyl)benzyl)carbamate (12). *tert*-Butyl (4-(((benzyloxy)carbonyl)amino)methyl)benzyl)carbamate (**10**, 519 mg, 1.40 mmol, 1.0 eq) was dissolved in CH₂Cl₂ (11 mL), treated with TFA (3 mL) and stirred for 1 h at room temperature. After evaporation of the solvent, the resulting solid was dissolved in EtOH (6 mL), DIPEA (0.730 mL, 542 mg, 4.20 mmol, 3.0 eq) and 2-chloropyrimidine-5-carbonitrile (390 mg, 2.80 mmol, 2.0 eq) were added and the reaction stirred at 80 °C for 24 h. The solvent was evaporated under reduced pressure, the residue was dissolved in EtOAc (100 mL), washed with brine (40 mL), dried over Na₂SO₄, filtered and concentrated under reduced pressure. The crude product was purified by silica gel column chromatography using cyclohexane/EtOAc (1+1) as eluent to afford **12** as a yellow solid (520 mg, 1.30 mmol).

Yield 92%; mp. 160-164 °C; *R*_f = 0.32 (cyclohexane/EtOAc (1+1)); ¹H NMR (600 MHz, DMSO-*d*₆) δ 4.15 (d, *J* = 6.2 Hz, 2H), 4.51 (d, *J* = 6.3 Hz, 2H), 5.02 (s, 2H), 7.18 (d, *J* = 7.9 Hz, 2H), 7.22 (d, *J* = 8.1 Hz, 2H), 7.25 – 7.34 (m, 1H), 7.32 – 7.37 (m, 4H), 7.75 (t, *J* = 6.2 Hz, 1H), 8.66 (d, *J* = 3.0 Hz, 1H), 8.68 (d, *J* = 3.0 Hz, 1H), 8.78 (t, *J* = 6.4 Hz, 1H); ¹³C NMR (126 MHz, DMSO-*d*₆) δ 43.7, 43.9, 65.5, 95.5, 117.2, 127.2, 127.2, 127.8, 127.9, 128.5, 137.3, 137.6, 138.5, 156.5, 161.5, 161.8; LRMS (ESI) *m/z* [M+H]⁺ calcd for C₂₁H₁₉N₅O₂ 374.2, found 374.2.



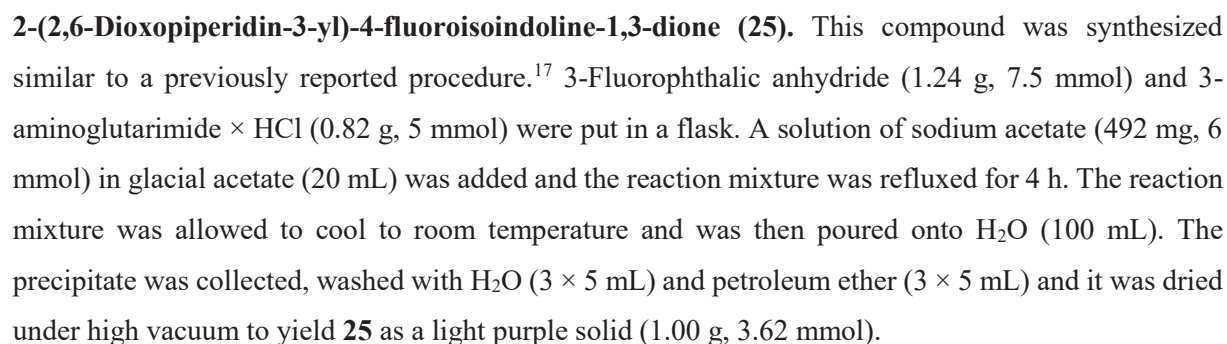
Benzyl (4-(((5-(1H-tetrazol-5-yl)pyrimidin-2-yl)amino)methyl)benzyl)carbamate (14). Benzyl (4-(((5-cyanopyrimidin-2-yl)amino)methyl)benzyl)carbamate (**12**, 281 mg, 0.750 mmol, 1.0 eq) was dissolved in DMF (3 mL). NaN₃ (97.5 mg, 1.50 mmol, 2.0 eq), NH₄Cl (52.4 mg, 0.980 mmol, 1.3 eq) and LiCl (16.0 mg, 0.380 mmol, 0.5 eq) were added and the reaction mixture was stirred at 100 °C for 18 h. After completion of the reaction, the mixture was quenched with ice water (9 mL) and acidified with 1 M HCl. The precipitated solid was filtered and washed with cold water to obtain the product **14** as a white solid (286 mg, 0.690 mmol).

Yield 92%; mp. 210-216 °C; ¹H NMR (500 MHz, DMSO-*d*₆) δ 4.17 (d, *J* = 6.1 Hz, 2H), 4.56 (d, *J* = 6.3 Hz, 2H), 5.03 (s, 2H), 7.20 (d, *J* = 7.9 Hz, 2H), 7.24 – 7.32 (m, 4H), 7.31 – 7.39 (m, 3H), 7.76 (t, *J* = 6.3 Hz, 1H), 8.41 (t, *J* = 6.3 Hz, 1H), 8.85 (s, 2H); ¹³C NMR (151 MHz, DMSO-*d*₆) δ 43.8, 44.0, 65.6, 108.1, 127.3, 127.3, 127.3, 128.0, 128.0, 128.6, 137.4, 138.3, 138.5, 156.6, 156.8, 157.1, 162.9; **LRMS (ESI)** *m/z* [M+H]⁺ calcd for C₂₁H₂₀N₈O₂ 417.2, found 417.2.

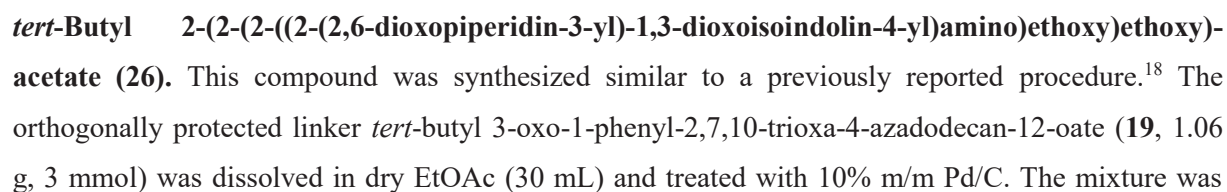


Benzyl (4-(((5-(5-(difluoromethyl)-1,3,4-oxadiazol-2-yl)pyrimidin-2-yl)amino)methyl)benzyl)carbamate (16). Benzyl (4-(((5-(1H-tetrazol-5-yl)pyrimidin-2-yl)amino)methyl)benzyl)carbamate (**14**, 286 mg, 0.690 mmol, 1.0 eq) was dissolved in toluene (7 mL). Difluoroacetic anhydride (DFAA, 0.260 mL, 364 mg, 2.07 mmol, 3.0 eq) was added and it was stirred at 70 °C for 18 h. The reaction mixture was concentrated under reduced pressure and purified by silica gel flash column chromatography using a CH₂Cl₂/MeOH gradient (0% to 5% MeOH) as eluent to yield **16** as a white solid (236 mg, 0.506 mmol).

Yield 72%; mp. 174-177 °C; *R*_f = 0.46 (CH₂Cl₂/MeOH (19+1)); ¹H NMR (500 MHz, DMSO-*d*₆) δ 4.17 (d, *J* = 6.2 Hz, 2H), 4.58 (d, *J* = 6.3 Hz, 2H), 5.03 (s, 2H), 7.20 (d, *J* = 7.9 Hz, 2H), 7.27 (d, *J* = 7.9 Hz, 2H), 7.28 – 7.39 (m, 5H), 7.50 (t, *J* = 51.6 Hz, 1H), 7.76 (t, *J* = 6.1 Hz, 1H), 8.70 (t, *J* = 6.4 Hz, 1H), 8.87 (s, 2H); ¹³C NMR (126 MHz, DMSO-*d*₆) δ 43.7, 44.0, 65.5, 106.5, 106.7 (t, *J*(C,F) = 237.6 Hz), 127.2, 127.2, 127.8, 127.9, 128.5, 137.3, 137.9, 138.5, 156.5, 157.1, 157.3, 157.7 (t, *J*(C,F) = 29.4 Hz), 163.1, 163.3; ¹⁹F NMR (565 MHz, DMSO-*d*₆) δ -121.1, -121.2; **HRMS (ESI)** *m/z* [M+H]⁺ calcd for C₂₃H₂₀F₂N₆O₃ 467.1638, found 467.1635; **HPLC** (95% H₂O 5 min, then to 95% MeCN in 5 min, then 100% MeCN to 20 min, 254 nm), *t*_R = 12.77 min, 98% purity.

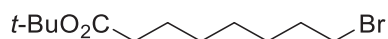


Yield 72%; $R_f = 0.67$ (petroleum ether/EtOAc (1+2)); mp. $> 230\text{ }^{\circ}\text{C}$, lit.¹⁷ mp. 236-238 $^{\circ}\text{C}$; **¹H NMR** (600 MHz, DMSO- d_6) δ 2.04 – 2.10 (m, 1H), 2.51 – 2.57 (m, 1H), 2.58 – 2.64 (m, 1H), 2.85 – 2.93 (m, 1H), 5.15 (dd, $J = 12.9, 5.4\text{ Hz}$, 1H), 7.73 (d, $J = 8.7\text{ Hz}$, 1H), 7.79 (d, $J = 7.3\text{ Hz}$, 1H), 7.92 – 7.97 (m, 1H), 11.13 (s, 1H); **¹³C NMR** (151 MHz, DMSO- d_6) δ 21.8, 30.9, 49.1, 117.0 (d, $^2J(\text{C},\text{F}) = 12.5\text{ Hz}$), 120.0 (d, $^4J(\text{C},\text{F}) = 2.8\text{ Hz}$), 123.0 (d, $^2J(\text{C},\text{F}) = 19.6\text{ Hz}$), 133.4, 138.0 (d, $^3J(\text{C},\text{F}) = 7.7\text{ Hz}$), 156.8 (d, $^1J(\text{C},\text{F}) = 262.3\text{ Hz}$), 163.9, 166.1 (d, $^3J(\text{C},\text{F}) = 2.6\text{ Hz}$), 169.6, 172.7; **LC-MS (ESI)** (90% H₂O + 2 mM NH₄OAc to 100% MeCN in 10 min, then 100% MeCN to 20 min, DAD 220-500 nm), $t_R = 6.99\text{ min}$, 100% purity, m/z $[\text{M}+\text{H}]^+$ calcd for C₁₃H₉FN₂O₄ 277.1, found 277.0.



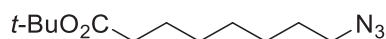
stirred under H₂ (1 atm, balloon) for 18 h. The mixture was filtered through celite and the filtrate was concentrated. The residue, compound **20**, was taken up in dry DMSO (30 mL). DIPEA (0.78 g, 6 mmol) and 2-(2,6-dioxopiperidin-3-yl)-4-fluoroisoindoline-1,3-dione (**25**, 0.83 g, 3 mmol) were added. The reaction mixture was stirred at 90 °C for 18 h. It was allowed to cool to room temperature. Then it was poured onto half-saturated brine (300 mL) and it was extracted with EtOAc (2 × 150 mL). The combined organic layers were washed with 5% aqueous LiCl solution (150 mL) and brine (150 mL), dried over Na₂SO₄, filtered and concentrated. The crude product was purified by silica gel column chromatography using a gradient from petroleum ether/EtOAc (1+1) to petroleum ether/EtOAc (1+2) to yield **26** as a yellow solid (581 mg, 1.22 mmol).

Yield 41%; *R*_f = 0.20 (petroleum ether/EtOAc (1+1)); mp. 66-68 °C, lit.¹⁷ mp. 66-68 °C; ¹H NMR (600 MHz, DMSO-*d*₆) δ 1.41 (s, 9H), 2.00 – 2.05 (m, 1H), 2.52 – 2.63 (m, 2H), 2.84 – 2.92 (m, 1H), 3.47 (q, *J* = 5.6 Hz, 2H), 3.59 (s, 4H), 3.63 (t, *J* = 5.5 Hz, 2H), 3.97 (s, 2H), 5.05 (dd, *J* = 12.8, 5.4 Hz, 1H), 6.60 (t, *J* = 5.8 Hz, 1H), 7.04 (d, *J* = 7.0 Hz, 1H), 7.15 (d, *J* = 8.6 Hz, 1H), 7.58 (dd, *J* = 8.5, 7.1 Hz, 1H), 11.07 (s, 1H); ¹³C NMR (151 MHz, DMSO-*d*₆) δ 22.1, 27.7, 31.0, 41.7, 48.5, 68.2, 68.9, 69.6, 69.9, 80.6, 109.2, 110.6, 117.4, 132.1, 136.2, 146.4, 167.3, 168.9, 169.3, 170.0, 172.7; **LC-MS (ESI)** (90% H₂O + 2 mM NH₄OAc to 100% MeOH + 2 mM NH₄OAc in 10 min, then 100% MeOH + 2 mM NH₄OAc to 20 min, DAD 220-500 nm), *t*_R = 10.19 min, 97% purity, *m/z* [M+H]⁺ calcd for C₂₃H₂₉N₃O₈ 476.2, found 476.2.



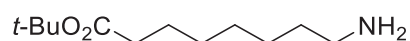
tert-Butyl 8-bromooctanoate (22). This compound was synthesized similar to a previously reported procedure.¹⁹ 8-Bromooctanoic acid (**21**, 4.46 g, 20 mmol) was dissolved in dry CH₂Cl₂ (50 mL) and cooled to 0 °C under nitrogen. Trifluoroacetic anhydride (4.62 g, 22 mmol) was added dropwise at 0 °C and the mixture was allowed to stir for 2.5 h. *tert*-Butanol (5.19 g, 70 mmol) was added dropwise at 0 °C and the mixture was stirred for 1 h at 0 °C. It was allowed to warm to room temperature and was stirred for 16 h. The reaction was quenched by the addition of water (50 mL). The organic phase was separated and the aqueous layer was extracted with CH₂Cl₂ (2 × 50 mL). The combined organic layers were washed with brine (50 mL), dried over Na₂SO₄, filtered and concentrated. The crude product was purified by silica gel column chromatography using petroleum ether/EtOAc (39+1) as eluent to yield **22** as a colourless liquid (4.09 g, 14.6 mmol).

Yield 73%; *R*_f = 0.43 (petroleum ether/EtOAc (39+1)); ¹H NMR (500 MHz, DMSO-*d*₆) δ 1.24 – 1.30 (m, 4H), 1.34 – 1.38 (m, 2H), 1.39 (s, 9H), 1.45 – 1.52 (m, 2H), 1.75 – 1.82 (m, 2H), 2.17 (t, *J* = 7.3 Hz, 2H), 3.52 (t, *J* = 6.7 Hz, 2H); ¹³C NMR (126 MHz, DMSO-*d*₆) δ 24.4, 27.3, 27.7, 27.7, 28.1, 32.1, 34.7, 35.0, 79.3, 172.2; **LC-MS (ESI)** (90% H₂O + 2 mM NH₄OAc to 100% MeCN in 10 min, then 100% MeCN to 15 min), *t*_R = 9.35 min, *m/z* [M+NH₄]⁺ calcd for C₁₂H₂₃BrO₂ 296.1, found 296.2.



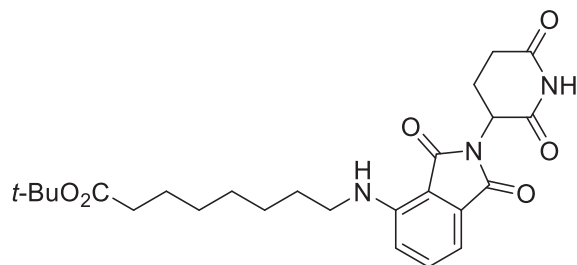
***tert*-Butyl 8-azidooctanoate (23).** *tert*-Butyl 8-bromooctanoate (**22**, 4.05 g, 14.5 mmol) was dissolved in dry DMF (20 mL). Sodium azide (1.13 g, 17.4 mmol) was added in one portion. The reaction mixture was stirred at 80 °C for 24 h. The reaction mixture was concentrated under high vacuum. The residue was diluted with water (30 mL). The aqueous phase was extracted with Et₂O (3 × 50 mL). The combined organic layers were washed with brine (50 mL), dried over Na₂SO₄, filtered and concentrated to yield **23** as a colourless oil (3.31 g, 13.7 mmol).

Yield 94%; *R*_f = 0.41 (petroleum ether/EtOAc (39+1)); ¹H NMR (500 MHz, DMSO-*d*₆) δ 1.23 – 1.33 (m, 6H), 1.39 (s, 9H), 1.44 – 1.56 (m, 4H), 2.17 (t, *J* = 7.3 Hz, 2H), 3.31 (t, *J* = 6.9 Hz, 2H); ¹³C NMR (126 MHz, DMSO-*d*₆) δ 24.4, 25.9, 27.7, 28.1, 28.1, 28.2, 34.7, 50.5, 79.2, 172.2.



***tert*-Butyl 8-aminooctanoate (24).** *tert*-Butyl 8-azidooctanoate (**23**, 3.26 g, 13.5 mmol) was dissolved in a mixture of THF (20 mL) and H₂O (10 mL). Triphenylphosphine (3.89 g, 14.9 mmol) was added in one portion and the reaction mixture was allowed to stir for 18 h at room temperature. The reaction mixture was diluted with H₂O (30 mL) and was extracted with CH₂Cl₂ (3 × 50 mL). The combined organic layers were dried over Na₂SO₄, filtered and concentrated. The crude product was purified by silica gel column chromatography using CH₂Cl₂ + 7 N NH₃ in MeOH (9+1) as eluent to yield **24** as a colourless liquid (2.47 g, 11.5 mmol).

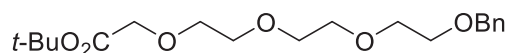
Yield 85%; *R*_f = 0.22 (CH₂Cl₂/7 N NH₃ in MeOH (19+1)); ¹H NMR (500 MHz, DMSO-*d*₆) δ 1.21 – 1.37 (m, 10H), 1.39 (s, 9H), 1.44 – 1.52 (m, 2H), 2.16 (t, *J* = 7.3 Hz, 2H), one signal (2H) is missing due to proton exchange; ¹³C NMR (126 MHz, DMSO-*d*₆) δ 24.5, 26.2, 27.7, 28.4, 28.6, 33.3, 34.7, 41.6, 79.2, 172.2; LC-MS (ESI) (90% H₂O + 2 mM NH₄OAc to 100% MeCN in 10 min, then 100% MeCN to 15 min), *t*_R = 5.12 min, *m/z* [M+H]⁺ calcd for C₁₂H₂₅NO₂ 216.2, found 216.2, lit.²⁰ *m/z* [M+H]⁺ found 216.2.



***tert*-Butyl 8-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisindolin-4-yl)amino)octanoate (27).** *tert*-Butyl 8-aminooctanoate (**24**, 646 mg, 3 mmol) was dissolved in dry DMF (10 mL). DIPEA (595 mg, 4.6 mmol) was added. 2-(2,6-Dioxopiperidin-3-yl)-4-fluoroisindoline-1,3-dione (**25**, 635 mg, 2.3 mmol) was added and the mixture was stirred at 90 °C for 18 h. The organic layer was concentrated under high

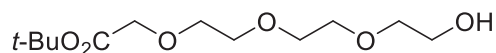
vacuum. The residue was purified by silica gel column chromatography using petroleum ether/EtOAc (2+1) as eluent to yield **27** as a yellow solid (383 mg, 0.81 mmol).

Yield 27%; R_f = 0.24 (petroleum ether/EtOAc (2+1)); mp. 68-70 °C; $^1\text{H NMR}$ (500 MHz, DMSO- d_6) δ 1.25 – 1.35 (m, 6H), 1.38 (s, 9H), 1.45 – 1.52 (m, 2H), 1.53 – 1.60 (m, 2H), 1.99 – 2.06 (m, 1H), 2.16 (t, J = 7.4 Hz, 2H), 2.53 – 2.62 (m, 2H), 2.83 – 2.93 (m, 1H), 5.04 (dd, J = 12.7, 5.4 Hz, 1H), 6.50 (t, J = 6.0 Hz, 1H), 7.02 (d, J = 7.0 Hz, 1H), 7.09 (d, J = 8.6 Hz, 1H), 7.57 (dd, J = 8.5, 7.2 Hz, 1H), 11.06 (s, 1H), one signal (2H) is obscured by the solvent signal; $^{13}\text{C NMR}$ (126 MHz, DMSO- d_6) δ 22.1, 24.5, 26.1, 27.7, 28.3, 28.3, 28.6, 30.9, 34.7, 41.8, 48.5, 79.3, 109.0, 110.3, 117.1, 132.1, 136.2, 146.4, 167.2, 168.9, 170.0, 172.2, 172.7; **LC-MS (ESI)** (90% H₂O + 2 mM NH₄OAc to 100% MeCN in 10 min, then 100% MeCN to 15 min, DAD 220-600 nm), t_R = 8.46 min, 100% purity, m/z [M-H]⁻ calcd for C₂₅H₃₃N₃O₆ 470.2, found 470.3.



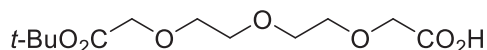
tert-Butyl 1-phenyl-2,5,8,11-tetraoxatridecan-13-oate (29). Potassium butoxide (1.18 g, 10.5 mmol) was dispersed in dry THF (60 mL) at 0 °C. 2-(2-(2-(Benzyloxy)ethoxy)ethoxy)ethan-1-ol (**28**, 2.40 g, 10 mmol) was added to the mixture. The solution was heated to 40 °C for 30 min. Afterwards the reaction was cooled to 0 °C. *tert*-Butyl 2-bromoacetate (1.95 g, 10 mmol) was added. The reaction mixture was stirred for 1 h at 0 °C. The mixture was allowed to warm to room temperature and was stirred for 18 h. The reaction mixture was partitioned between water (100 mL) and EtOAc (100 mL). The organic layer was separated. The aqueous phase was extracted with EtOAc (2 × 100 mL). The combined organic layers were washed with brine (100 mL), dried over Na₂SO₄, filtered and concentrated. The residue was purified by silica gel column chromatography using a gradient from petroleum ether/EtOAc (4+1) to petroleum ether/EtOAc (2+1) to yield **29** as a colourless oil (1.21 g, 3.41 mmol).

Yield 34%; R_f = 0.30 (petroleum ether/EtOAc (2+1)); $^1\text{H NMR}$ (500 MHz, DMSO- d_6) δ 1.42 (s, 9H), 3.51 – 3.57 (m, 12H), 3.97 (s, 2H), 4.49 (s, 2H), 7.25 – 7.37 (m, 5H); $^{13}\text{C NMR}$ (126 MHz, DMSO- d_6) δ 27.7, 68.1, 69.1, 69.7, 69.7, 69.8, 69.8, 72.0, 80.6, 127.3, 127.4, 128.1, 138.5, 169.3, one signal is missing due to overlapping peaks; **LC-MS (ESI)** (90% H₂O + 2 mM NH₄OAc to 100% MeOH + 2 mM NH₄OAc in 10 min, then 100% MeOH + 2 mM NH₄OAc to 20 min), t_R = 11.12 min, 90% purity, m/z [M+NH₄]⁺ calcd for C₁₉H₃₀O₆ 372.2, found 372.2.



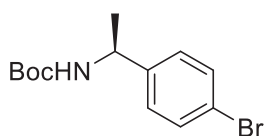
tert-Butyl 2-(2-(2-(2-hydroxyethoxy)ethoxy)ethoxy)acetate (30). This compound was synthesized similar to a previously reported procedure.²¹ *tert*-Butyl 1-phenyl-2,5,8,11-tetraoxatridecan-13-oate (**29**, 1.24 g, 3.5 mmol) was dissolved in dry EtOH (10 mL) and treated with 10% m/m Pd/C under H₂ (1 atm, balloon) and was stirred for 18 h. The mixture was diluted with EtOAc (20 mL), filtered through celite and concentrated to yield **30** as a colourless oil.

Quantitative yield; $R_f = 0.48$ ($\text{CH}_2\text{Cl}_2/\text{MeOH}$ (9+1)); $^1\text{H NMR}$ (500 MHz, $\text{DMSO}-d_6$) δ 1.41 – 1.43 (m, 9H), 3.40 – 3.43 (m, 2H), 3.47 – 3.59 (m, 10H), 3.97 – 3.99 (m, 2H), 4.51 – 4.55 (m, 1H); $^{13}\text{C NMR}$ (126 MHz, $\text{DMSO}-d_6$) δ 27.7, 60.2, 68.1, 69.7, 69.7, 69.7, 69.8, 72.3, 80.6, 169.3; **LC-MS (ESI)** (90% H_2O + 2 mM NH_4OAc to 100% MeOH + 2 mM NH_4OAc in 10 min, then 100% MeOH + 2 mM NH_4OAc to 20 min), $t_R = 8.58$ min, m/z $[\text{M}+\text{NH}_4]^+$ calcd for $\text{C}_{12}\text{H}_{24}\text{O}_6$ 282.2, found 282.2.



13,13-Dimethyl-11-oxo-3,6,9,12-tetraoxatetradecanoic acid (31). This compound was synthesized similar to a previously reported procedure.²¹ *tert*-Butyl 2-(2-(2-(2-hydroxyethoxy)ethoxy)ethoxy)acetate (**30**, 925 mg, 3.5 mmol) was dissolved in MeCN (6 mL) and water (6 mL). TEMPO (120 mg, 0.77 mmol) was added. (Diacetoxyiodo)benzene (2.48 g, 7.7 mmol) was added portionwise. The reaction mixture was stirred at room temperature for 16 h. It was quenched by the addition of saturated NaHCO_3 solution (85 mL) and the aqueous layer was washed with EtOAc (2×50 mL). The aqueous phase was acidified with 2 N HCl until pH~1. The mixture was then extracted with EtOAc (2×50 mL) and the combined organic layers were dried over Na_2SO_4 , filtered and concentrated. The residue was purified by silica gel column chromatography using a gradient of CH_2Cl_2 to $\text{CH}_2\text{Cl}_2/\text{MeOH}$ (19+1) to yield **31** as a colourless oil (460 mg, 1.65 mmol).

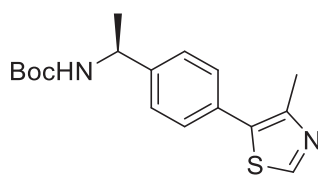
Yield 47%; $R_f = 0.19$ ($\text{CH}_2\text{Cl}_2/\text{MeOH}$ (9+1)); $^1\text{H NMR}$ (500 MHz, $\text{DMSO}-d_6$) δ 1.42 (s, 9H), 3.52 – 3.59 (m, 8H), 3.98 (s, 2H), 4.01 (s, 2H), one signal (1H) is missing due to proton exchange; $^{13}\text{C NMR}$ (126 MHz, $\text{DMSO}-d_6$) δ 27.7, 67.6, 68.1, 69.6, 69.7, 69.8, 69.8, 80.6, 169.3, 171.6; **LC-MS (ESI)** (90% H_2O + 0.1% AcOH to 100% MeCN + 0.1% AcOH in 10 min, then 100% MeCN + 0.1% AcOH to 20 min), $t_R = 6.22$ min, m/z $[\text{M}+\text{NH}_4]^+$ calcd for $\text{C}_{12}\text{H}_{22}\text{O}_7$ 296.2, found 296.2.



***tert*-Butyl (S)-(1-(4-bromophenyl)ethyl)carbamate (37).** This compound was synthesized similar to a previously reported procedure.²² (*S*)-1-(4-Bromophenyl)ethan-1-amine (**36**, 5.00 g, 25 mmol) and NaHCO_3 (1.58 g, 18.8 mmol) were dissolved in H_2O (15 mL) and EtOAc (10 mL) and cooled to 0 °C. Boc_2O (6.55 g, 30 mmol) was dissolved in EtOAc (3 mL) and was added dropwise. The reaction mixture was stirred for 2 h at 0 °C. The precipitate was collected, resuspended in $\text{H}_2\text{O}/\text{hexane}$ (1+1) (25 mL), stirred for further 30 min and was again collected. The residue was washed with hexane (3×15 mL) and dried under high vacuum to yield **37** as a white solid (6.90 g, 23 mmol).

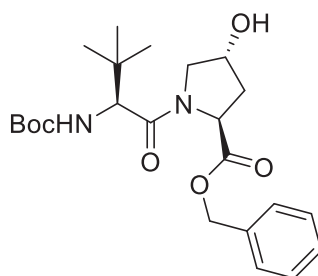
Yield 92%; mp. 141-143 °C; $R_f = 0.71$ (petroleum ether/EtOAc (4+1)); $^1\text{H NMR}$ (600 MHz, $\text{DMSO}-d_6$) δ 1.27 (d, $J = 7.0$ Hz, 3H), 1.35 (s, 9H), 4.53 – 4.62 (m, 1H), 7.24 (d, $J = 8.3$ Hz, 2H), 7.39 (d, $J = 8.2$ Hz, 1H), 7.47 – 7.51 (m, 2H); $^{13}\text{C NMR}$ (151 MHz, $\text{DMSO}-d_6$) δ 22.6, 28.2, 49.1, 77.7, 119.4, 128.1, 131.0, 144.9, 154.7; **LC-MS (ESI)** (90% H_2O + 2 mM NH_4OAc to 100% MeCN in 10 min, then 100%

MeCN to 20 min, DAD 205-400 nm), t_R = 9.86 min, 100% purity, m/z $[M+H]^+$ calcd for $C_{13}H_{18}BrNO_2$ 302.1, found 302.0.



tert-Butyl (S)-1-(4-(4-methylthiazol-5-yl)phenyl)ethylcarbamate (38). This compound was synthesized similar to a previously reported procedure.²² *tert*-Butyl (S)-1-(4-bromophenyl)ethylcarbamate (**37**, 6.00 g, 20 mmol), $Pd(OAc)_2$ (45 mg, 0.2 mmol) and KOAc (3.93 g, 40 mmol) were dissolved in dry DMA (20 mL). 4-Methylthiazole (3.97 g, 40 mmol) was added and the mixture was heated to 130 °C under argon for 4 h. The mixture was allowed to cool to room temperature and was concentrated under high vacuum. The residue was diluted with water (80 mL) and was extracted with CH_2Cl_2 (3×80 mL). The combined organic layers were washed with brine (80 mL), dried over Na_2SO_4 , filtered and concentrated. The residue was purified by silica gel column chromatography using petroleum ether/EtOAc (4+1) as eluent to yield **38** as a white solid (5.34 g, 16.8 mmol).

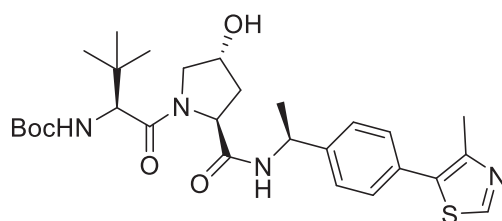
Yield 84%; mp. 124-126 °C; R_f = 0.15 (petroleum ether/EtOAc (4+1)); ^1H NMR (500 MHz, $DMSO-d_6$) δ 1.33 (d, J = 7.0 Hz, 3H), 1.37 (s, 9H), 2.45 (s, 3H), 4.60 – 4.71 (m, 1H), 7.37 – 7.46 (m, 5H), 8.97 (s, 1H); ^{13}C NMR (126 MHz, $DMSO-d_6$) δ 15.9, 22.7, 28.2, 49.2, 77.7, 126.3, 128.7, 129.6, 131.1, 145.3, 147.7, 151.3, 154.8; LC-MS (ESI) (90% H_2O + 2 mM NH_4OAc to 100% MeOH + 2 mM NH_4OAc in 10 min, then 100% MeOH + 2 mM NH_4OAc to 20 min, DAD 220-400 nm), t_R = 11.11 min, 96% purity, m/z $[M+H]^+$ calcd for $C_{17}H_{22}N_2O_2S$ 319.1, found 318.9.



Benzyl (2S,4R)-1-((S)-2-((tert-butoxycarbonyl)amino)-3,3-dimethylbutanoyl)-4-hydroxy-pyrrolidine-2-carboxylate (41). Boc-Tle-OH (**39**, 4.63 g, 20 mmol) was dissolved in dry DMF (18 mL). HATU (8.03 g, 21.1 mmol) and DIPEA (9.05 g, 70 mmol) were added at 0°C and the mixture was stirred for 30 min under nitrogen. H-Hyp-OBzl $\times$ HCl (**40**, 5.15 g, 20 mmol) was dissolved in dry DMF (18 mL) and was added to the reaction mixture. The mixture was stirred under nitrogen at room temperature for 18 h. The reaction was quenched by the addition of H_2O (50 mL) and was extracted with EtOAc (3×50 mL). The combined organic layers were washed with saturated $NaHCO_3$ solution (50 mL) and brine (50 mL), dried over Na_2SO_4 , filtered and concentrated. The residue was purified by

silica gel column chromatography using petroleum ether/EtOAc (1+1) as eluent to yield **41** as a white solid (5.91 g, 13.6 mmol).

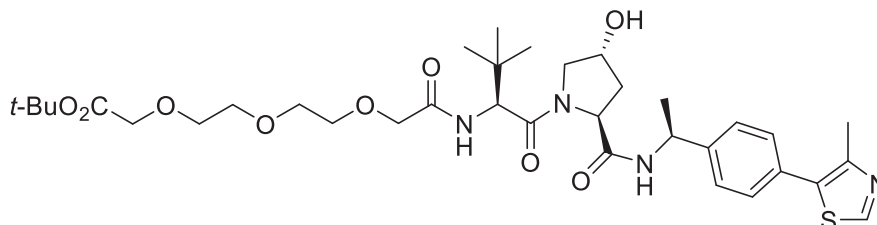
Yield 68%; mp. 118-120 °C; R_f = 0.33 (petroleum ether/EtOAc (1+1)); $^1\text{H NMR}$ (500 MHz, DMSO- d_6) δ 0.89 (s, 9H), 1.38 (s, 9H), 1.88 – 1.96 (m, 1H), 2.10 – 2.16 (m, 1H), 3.58 – 3.64 (m, 1H), 3.67 (dd, J = 10.6, 4.1 Hz, 1H), 4.15 (d, J = 9.4 Hz, 1H), 4.34 (br s, 1H), 4.42 (t, J = 8.3 Hz, 1H), 5.06 – 5.15 (m, 2H), 5.19 (d, J = 3.8 Hz, 1H), 6.47 (d, J = 9.4 Hz, 1H), 7.29 – 7.39 (m, 5H); $^{13}\text{C NMR}$ (126 MHz, DMSO- d_6) δ 26.1, 28.1, 35.1, 37.2, 55.9, 57.7, 58.2, 65.8, 68.7, 78.1, 127.8, 127.9, 128.3, 135.8, 155.3, 170.1, 171.6; **LC-MS (ESI)** (90% H₂O + 2 mM NH₄OAc to 100% MeOH + 2 mM NH₄OAc in 10 min, then 100% MeOH + 2 mM NH₄OAc to 20 min, DAD 200-400 nm), t_R = 11.41 min, 100% purity, m/z [M+H]⁺ calcd for C₂₃H₃₄N₂O₆ 435.2, found 435.4.



***tert*-Butyl ((*S*)-1-((2*S*,4*R*)-4-hydroxy-2-(((*S*)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)carbonyl)-pyrrolidin-1-yl)-3,3-dimethyl-1-oxobutan-2-yl)carbamate (**33**)**. This compound was synthesized similar to a previously reported procedure.²³ Benzyl (2*S*,4*R*)-1-((*S*)-2-((*tert*-butoxycarbonyl)amino)-3,3-dimethylbutanoyl)-4-hydroxypyrrolidine-2-carboxylate (**41**, 2.17 g, 5 mmol) was dissolved in dry EtOH (50 mL) and treated with 10% m/m Pd/C under H₂ (1 atm, balloon) for 18 h. The reaction mixture was filtered through celite and was concentrated to yield a white solid. *tert*-Butyl (*S*)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)carbamate (**38**, 1.59 g, 5 mmol) was dissolved in dry CH₂Cl₂ (15 mL) and TFA (5 mL) was added. The mixture was stirred for 2 h at room temperature. The mixture was concentrated under high vacuum. The obtained intermediate from the hydrogenolytic deprotection (2*S*,4*R*)-1-((*S*)-2-((*tert*-Butoxycarbonyl)amino)-3,3-dimethylbutanoyl)-4-hydroxy-pyrrolidine-2-carboxylic acid (1.72 g, 5 mmol) was dissolved in dry DMF (5 mL) and HATU (2.09 g, 5.5 mmol) and DIPEA (2.26 g, 17.5 mmol) were added. The concentrated residue of the TFA mediated deprotection was dissolved in dry DMF (5 mL) and was added to the reaction mixture. The reaction mixture was stirred at room temperature under argon for 18 h. The reaction was quenched by the addition of H₂O (25 mL) and was then extracted with EtOAc (3 × 50 mL). The combined organic layers were washed with brine (75 mL), dried over Na₂SO₄, filtered and concentrated. The residue was purified by silica gel column chromatography using EtOAc as eluent to yield **33** as a white solid (1.75 g, 3.21 mmol).

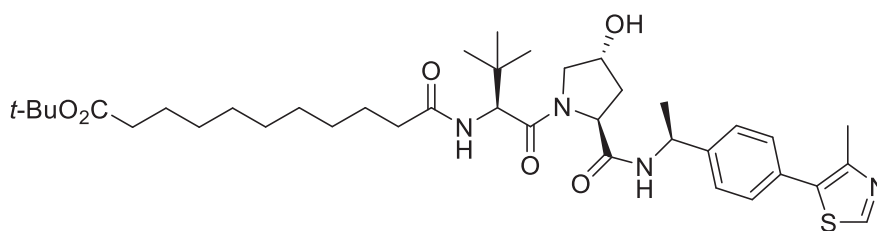
Yield 64%; mp. 208-210 °C; R_f = 0.29 (EtOAc); $^1\text{H NMR}$ (600 MHz, DMSO- d_6) δ 0.93 (s, 9H), 1.36 – 1.40 (m, 12H), 1.75 – 1.82 (m, 1H), 2.00 – 2.05 (m, 1H), 2.45 (s, 3H), 3.52 – 3.63 (m, 2H), 4.14 (d, J = 9.3 Hz, 1H), 4.28 (br s, 1H), 4.45 (t, J = 8.0 Hz, 1H), 4.87 – 4.93 (m, 1H), 5.10 (d, J = 3.6 Hz, 1H), 6.37 (d, J = 9.3 Hz, 1H), 7.36 – 7.39 (m, 2H), 7.42 – 7.44 (m, 2H), 8.38 (d, J = 7.6 Hz, 1H), 8.98 (s, 1H); $^{13}\text{C NMR}$ (151 MHz, DMSO- d_6) δ 15.9, 22.4, 26.3, 28.2, 35.3, 37.7, 47.7, 56.2, 58.4, 58.5, 68.8, 78.1, 126.3,

128.8, 129.6, 131.1, 144.7, 147.7, 151.4, 155.3, 169.7, 170.6; **LC-MS (ESI)** (90% H₂O + 2 mM NH₄OAc to 100% MeOH + 2 mM NH₄OAc in 10 min, then 100% MeOH + 2 mM NH₄OAc to 20 min, DAD 220-400 nm), *t_R* = 11.32 min, 97% purity, *m/z* [M+H]⁺ calcd for C₂₈H₄₀N₄O₅S 545.3, found 545.1.



***tert*-Butyl ((*S*)-13-((2*S*,4*R*)-4-hydroxy-2-(((*S*)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)carbamoyl)-pyrrolidine-1-carbonyl)-14,14-dimethyl-11-oxo-3,6,9-trioxa-12-azapentadecanoate (**34**).** *tert*-Butyl ((*S*)-1-((2*S*,4*R*)-4-hydroxy-2-(((*S*)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)carbamoyl)pyrrolidin-1-yl)-3,3-dimethyl-1-oxobutan-2-yl)carbamate (**33**, 872 mg, 1.6 mmol) was dissolved in dry CH₂Cl₂ (15 mL) and was treated with TFA (5 mL). The mixture was stirred for 2 h at room temperature. The mixture was concentrated under high vacuum. 13,13-Dimethyl-11-oxo-3,6,9,12-tetraoxatetradecanoic acid (**31**, 445 mg, 1.6 mmol) was dissolved in dry DMF (5 mL). HATU (669 mg, 1.76 mmol) and DIPEA (724 mg, 5.6 mmol) were added under argon. The deprotected VHL-ligand was dissolved in dry DMF (5 mL) and was added to the mixture, containing the activated acid compound. The reaction mixture was allowed to stir at room temperature under argon for 18 h. The reaction mixture was concentrated under high vacuum. The residue was diluted with water (25 mL) and was extracted with EtOAc (3 × 50 mL). The combined organic layers were washed with brine (75 mL), dried over Na₂SO₄, filtered and concentrated. The crude product was purified by silica gel column chromatography using CH₂Cl₂/MeOH (19+1) as eluent to yield **34** as a colourless resin (902 mg, 1.28 mmol).

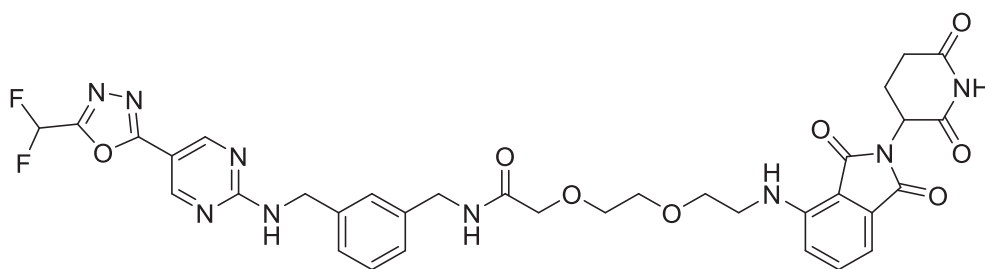
Yield 80%; *R_f* = 0.24 (CH₂Cl₂/MeOH (19+1)); ¹H NMR (600 MHz, DMSO-*d*₆) δ 0.94 (s, 9H), 1.38 (d, *J* = 7.0 Hz, 3H), 1.42 (s, 9H), 1.75 – 1.82 (m, 1H), 2.01 – 2.07 (m, 1H), 2.45 (s, 3H), 3.53 – 3.65 (m, 10H), 3.96 (d, *J* = 1.9 Hz, 2H), 3.98 (s, 2H), 4.28 (br s, 1H), 4.45 (t, *J* = 8.2 Hz, 1H), 4.55 (d, *J* = 9.5 Hz, 1H), 4.88 – 4.94 (m, 1H), 5.11 (d, *J* = 3.5 Hz, 1H), 7.34 – 7.39 (m, 3H), 7.41 – 7.46 (m, 2H), 8.41 (d, *J* = 7.7 Hz, 1H), 8.98 (s, 1H); ¹³C NMR (151 MHz, DMSO-*d*₆) δ 15.9, 22.4, 26.2, 27.7, 35.7, 37.7, 47.7, 55.7, 56.5, 58.5, 68.1, 68.7, 69.5, 69.6, 69.7, 69.9, 70.4, 80.6, 126.3, 128.8, 129.7, 131.1, 144.7, 147.7, 151.4, 168.5, 169.0, 169.3, 170.4; **LC-MS (ESI)** (90% H₂O + 2 mM NH₄OAc to 100% MeOH + 2 mM NH₄OAc in 10 min, then 100% MeOH + 2 mM NH₄OAc to 20 min, DAD 220-400 nm), *t_R* = 11.47 min, 98% purity, *m/z* [M+H]⁺ calcd for C₃₅H₅₂N₄O₉S 705.4, found 705.6.



tert-Butyl 11-(((S)-1-((2S,4R)-4-hydroxy-2-(((S)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)carbamoyl)pyrrolidin-1-yl)-3,3-dimethyl-1-oxobutan-2-yl)amino)-11-oxoundecanoate (35). *tert*-Butyl

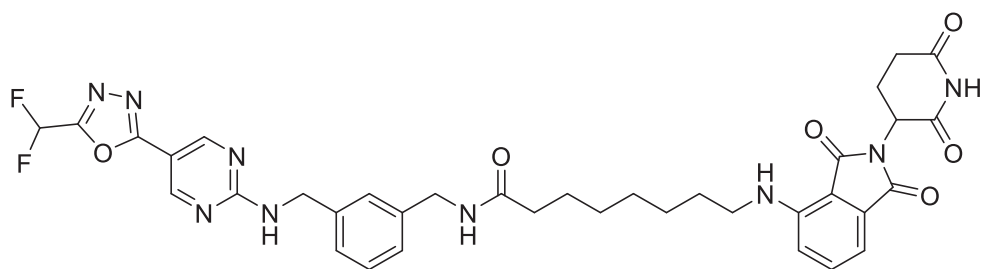
((S)-1-((2S,4R)-4-hydroxy-2-(((S)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)carbamoyl)-pyrrolidin-1-yl)-3,3-dimethyl-1-oxobutan-2-yl)carbamate (**33**, 490 mg, 0.9 mmol) was dissolved in dry CH₂Cl₂ (5 mL) and was treated with TFA (5 mL). The mixture was stirred for 2 h at room temperature. The mixture was concentrated under high vacuum. 11-(*tert*-Butoxy)-11-oxoundecanoic acid (**32**, 245 mg, 0.9 mmol) was dissolved in dry DMF (5 mL). HATU (376 mg, 0.99 mmol) and DIPEA (407 mg, 3.15 mmol) were added under argon. The deprotected VHL ligand was dissolved in dry DMF (5 mL) and was added to the mixture containing the activated acid compound. The reaction mixture was allowed to stir at room temperature under argon for 3 h. The reaction mixture was concentrated under high vacuum. The residue was diluted with water (50 mL) and was extracted with CH₂Cl₂ (3 × 50 mL). The combined organic layers were washed with brine (50 mL), dried over Na₂SO₄, filtered and concentrated. The crude product was purified by silica gel column chromatography using CH₂Cl₂/MeOH (19+1) as eluent to yield **35** as a colourless resin (441 mg, 0.63 mmol).

Yield 70%; *R*_f = 0.28 (CH₂Cl₂/MeOH (9+1)); ¹H NMR (600 MHz, DMSO-*d*₆) δ 0.93 (s, 9H), 1.22 – 1.25 (m, 10H), 1.37 – 1.39 (m, 12H), 1.43 – 1.51 (m, 4H), 1.77 – 1.83 (m, 1H), 1.97 – 2.03 (m, 1H), 2.06 – 2.13 (m, 1H), 2.14 – 2.18 (m, 2H), 2.21 – 2.28 (m, 1H), 2.45 (s, 3H), 3.57 – 3.64 (m, 2H), 4.26 – 4.30 (m, 1H), 4.42 (t, *J* = 8.0 Hz, 1H), 4.52 (d, *J* = 9.3 Hz, 1H), 4.89 – 4.95 (m, 1H), 5.07 (d, *J* = 3.5 Hz, 1H), 7.38 (d, *J* = 8.2 Hz, 2H), 7.43 (d, *J* = 8.2 Hz, 2H), 7.75 (d, *J* = 9.3 Hz, 1H), 8.34 (d, *J* = 7.8 Hz, 1H), 8.98 (s, 1H); ¹³C NMR (151 MHz, DMSO-*d*₆) δ 15.9, 22.4, 24.6, 25.4, 26.4, 27.7, 28.3, 28.6, 28.6, 28.7, 34.7, 34.9, 35.2, 37.7, 38.2, 47.7, 56.2, 56.3, 58.5, 68.7, 79.3, 126.4, 128.8, 129.7, 131.1, 144.6, 147.7, 151.4, 169.6, 170.6, 172.0, 172.3; LC-MS (ESI) (90% H₂O + 2 mM NH₄OAc to 100% MeCN in 10 min, then 100% MeCN to 15 min, DAD 220–600 nm), *t*_R = 8.48 min, 95% purity, *m/z* [M+H]⁺ calcd for C₃₈H₅₈N₄O₆S 699.4, found 699.6.



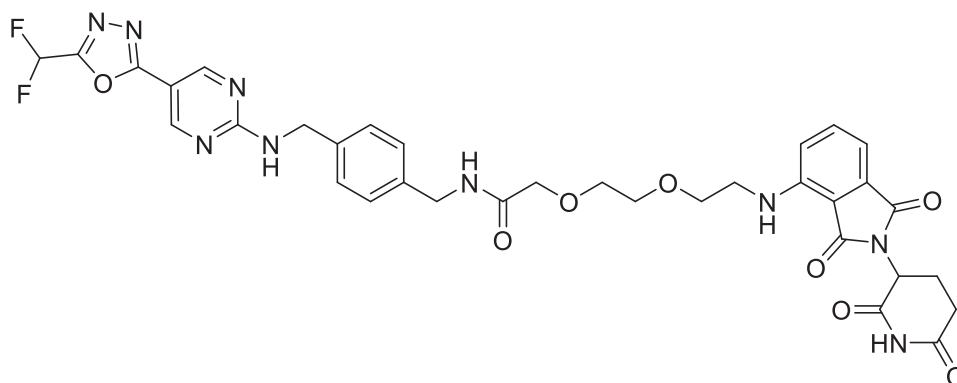
***N*-(3-(((5-(5-(Difluoromethyl)-1,3,4-oxadiazol-2-yl)pyrimidin-2-yl)amino)methyl)benzyl)-2-(2-(2-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisindolin-4-yl)amino)ethoxy)ethoxy)acetamide (1).** Benzyl (3-(((5-(5-(difluoromethyl)-1,3,4-oxadiazol-2-yl)pyrimidin-2-yl)amino)methyl)benzyl)carbamate (**15**, 117 mg, 0.25 mmol) was dissolved in a mixture of dry EtOH (10 mL) and dry EtOAc (5 mL). The mixture was treated with 10% m/m Pd/C under H₂ (1 atm, balloon) for 18 h. The mixture was filtered through celite and concentrated under high vacuum. *tert*-Butyl 2-(2-(2-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisindolin-4-yl)amino)ethoxy)ethoxy)acetate (**26**, 119 mg, 0.25 mmol) was dissolved in dry CH₂Cl₂ (5 mL) and was treated with TFA (5 mL) for 2 h at room temperature. The mixture was concentrated under high vacuum. The deprotected acid compound was dissolved in dry DMF (5 mL). DIPEA (97 mg, 0.75 mmol) and HATU (105 mg, 0.28 mmol) were added under argon. The deprotected amine compound was dissolved in dry DMF (5 mL) and was added to the reaction mixture. The reaction mixture was allowed to stir at room temperature under argon for 2 h. The reaction mixture was concentrated under high vacuum. The crude product was purified by silica gel flash column chromatography using a CH₂Cl₂/MeOH gradient (0% to 4% MeOH) to yield **1** as a yellow solid (49 mg, 0.07 mmol).

Yield 27%; mp. 106-110 °C; *R*_f = 0.31 (CH₂Cl₂/MeOH (19+1)); ¹H NMR (600 MHz, DMSO-*d*₆) δ 1.99 – 2.05 (m, 1H), 2.52 – 2.62 (m, 2H), 2.82 – 2.90 (m, 1H), 3.40 – 3.43 (m, 2H), 3.58 – 3.61 (m, 6H), 3.93 (s, 2H), 4.28 (d, *J* = 6.2 Hz, 2H), 4.57 (d, *J* = 6.3 Hz, 2H), 5.03 (dd, *J* = 12.8, 5.4 Hz, 1H), 6.56 (t, *J* = 5.9 Hz, 1H), 7.02 (d, *J* = 7.0 Hz, 1H), 7.08 – 7.13 (m, 2H), 7.16 – 7.26 (m, 3H), 7.51 (t, *J* = 51.3 Hz, 1H), 7.53 – 7.57 (m, 1H), 8.15 (t, *J* = 6.2 Hz, 1H), 8.70 (t, *J* = 6.4 Hz, 1H), 8.86 (s, 2H), 11.07 (s, 1H); ¹³C NMR (151 MHz, DMSO-*d*₆) δ 22.1, 31.0, 41.6, 41.6, 44.0, 48.5, 68.9, 69.4, 70.0, 70.3, 106.3, 106.5 (t, ¹*J* (C,F) = 238.6 Hz), 109.2, 110.6, 117.3, 125.5, 125.7, 125.9, 128.2, 132.0, 136.2, 139.2, 139.5, 146.3, 157.0, 157.1, 157.5 (t, ²*J* (C,F) = 29.4 Hz), 162.9, 163.1, 167.2, 168.9, 169.2, 170.0, 172.7; LC-MS (ESI) (90% H₂O + 2 mM NH₄OAc to 100% MeCN in 10 min, then 100% MeCN to 15 min, DAD 200-600 nm), *t*_R = 5.84 min, 96% purity, *m/z* [M+H]⁺ calcd for C₃₄H₃₃F₂N₉O₈ 734.2, found 734.4; HRMS (ESI) *m/z* [M+H]⁺ calcd for C₃₄H₃₃F₂N₉O₈ 734.2493, found 734.2492.



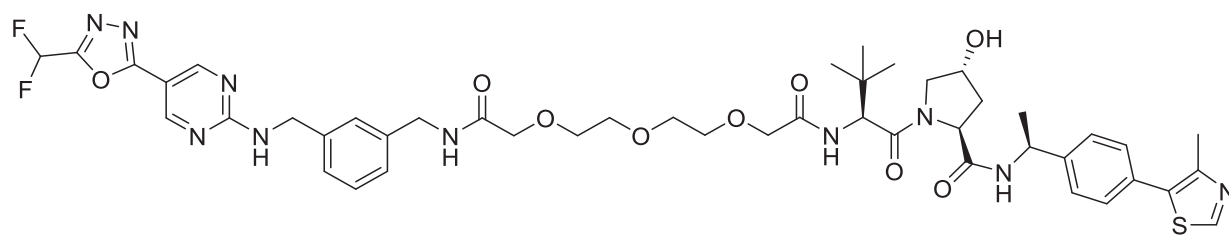
***N*-(3-(((5-(5-(Difluoromethyl)-1,3,4-oxadiazol-2-yl)pyrimidin-2-yl)amino)methyl)benzyl)-8-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisindolin-4-yl)amino)octanamide (2).** Benzyl 3-(((5-(5-(difluoromethyl)-1,3,4-oxadiazol-2-yl)pyrimidin-2-yl)amino)methyl)benzyl)carbamate (**15**, 93 mg, 0.2 mmol) was dissolved in dry THF (10 mL). The mixture was treated with 10% m/m Pd/C under H₂ (1 atm, balloon) for 18 h. The mixture was filtered through celite and concentrated under high vacuum. *tert*-Butyl 8-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisindolin-4-yl)amino)octanoate (**27**, 94 mg, 0.2 mmol) was dissolved in dry CH₂Cl₂ (5 mL) and was treated with TFA (5 mL) for 2 h at room temperature. The mixture was concentrated under high vacuum. The deprotected acid compound was dissolved in dry DMF (5 mL). DIPEA (78 mg, 0.6 mmol) and HATU (84 mg, 0.22 mmol) were added under argon. The deprotected amine compound was dissolved in dry DMF (5 mL) and was added to the reaction mixture. The reaction mixture was allowed to stir at room temperature under argon for 2 h. The reaction mixture was concentrated under high vacuum. The crude product was purified by silica gel flash column chromatography using a CH₂Cl₂/MeOH gradient (0% to 4% MeOH) to yield **2** as a yellow solid (36 mg, 0.05 mmol).

Yield 25%; mp. 108-112 °C; *R*_f = 0.33 (CH₂Cl₂/MeOH (19+1)); ¹H NMR (600 MHz, DMSO-*d*₆) δ 1.22 – 1.34 (m, 6H), 1.46 – 1.52 (m, 2H), 1.52 – 1.59 (m, 2H), 2.00 – 2.05 (m, 1H), 2.10 (t, *J* = 7.4 Hz, 2H), 2.52 – 2.62 (m, 2H), 2.84 – 2.91 (m, 1H), 3.25 – 3.30 (m, 2H), 4.22 (d, *J* = 5.9 Hz, 2H), 4.58 (d, *J* = 6.3 Hz, 2H), 5.04 (dd, *J* = 12.8, 5.4 Hz, 1H), 6.50 (t, *J* = 6.0 Hz, 1H), 7.01 (d, *J* = 7.0 Hz, 1H), 7.06 – 7.12 (m, 2H), 7.16 – 7.21 (m, 2H), 7.25 (t, *J* = 7.5 Hz, 1H), 7.52 (t, *J* = 51.3 Hz, 1H), 7.56 (dd, *J* = 8.5, 7.1 Hz, 1H), 8.24 (t, *J* = 6.1 Hz, 1H), 8.71 (t, *J* = 6.4 Hz, 1H), 8.87 (s, 2H), 11.07 (s, 1H); ¹³C NMR (151 MHz, DMSO-*d*₆) δ 22.1, 25.2, 26.2, 28.4, 28.6, 28.6, 31.0, 35.3, 41.8, 41.9, 44.0, 48.5, 106.3, 106.5 (t, ¹*J*(C,F) = 238.2 Hz), 109.0, 110.3, 117.1, 125.4, 125.7, 125.8, 128.2, 132.2, 136.2, 139.2, 139.9, 146.4, 157.0, 157.1, 157.5 (t, ²*J*(C,F) = 29.5 Hz), 162.9, 163.1, 167.3, 168.9, 170.0, 172.0, 172.8; LC-MS (ESI) (90% H₂O + 2 mM NH₄OAc to 100% MeCN in 10 min, then 100% MeCN to 15 min, DAD 220-600 nm), *t*_R = 6.99 min, 98% purity, *m/z* [M+H]⁺ calcd for C₃₆H₃₇F₂N₉O₆ 730.3, found 730.5; HRMS (ESI) *m/z* [M+H]⁺ calcd for C₃₆H₃₇F₂N₉O₆ 730.2908, found 730.2907.



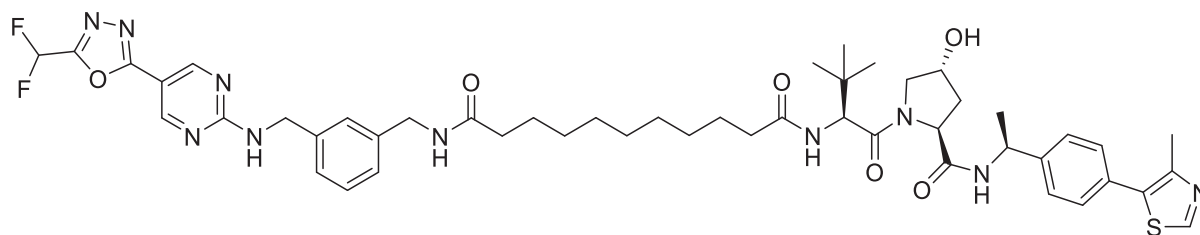
***N*-(4-(((5-(5-(Difluoromethyl)-1,3,4-oxadiazol-2-yl)pyrimidin-2-yl)amino)methyl)benzyl)-2-(2-(2-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisindolin-4-yl)amino)ethoxy)ethoxy)acetamide (3).** Benzyl (4-(((5-(5-(difluoromethyl)-1,3,4-oxadiazol-2-yl)pyrimidin-2-yl)amino)methyl)benzyl)carbamate (**16**, 117 mg, 0.25 mmol) was dissolved in a mixture of dry EtOAc (10 mL), dry EtOH (2 mL) and dry THF (2 mL). The mixture was treated with 10% m/m Pd/C under H₂ (1 atm, balloon) for 4 days. The mixture was filtered through celite and concentrated under high vacuum. *tert*-Butyl 2-(2-(2-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisindolin-4-yl)amino)ethoxy)ethoxy)acetate (**26**, 119 mg, 0.25 mmol) was dissolved in dry CH₂Cl₂ (5 mL) and was treated with TFA (5 mL) for 2 h at room temperature. The mixture was concentrated under high vacuum. The deprotected acid compound was dissolved in dry DMF (5 mL). DIPEA (97 mg, 0.75 mmol) and HATU (105 mg, 0.28 mmol) were added under argon. The deprotected amine compound was dissolved in dry DMF (5 mL) and was added to the reaction mixture. The reaction mixture was allowed to stir at room temperature under argon for 2 h. The reaction mixture was concentrated under high vacuum. The crude product was purified by silica gel flash column chromatography using a CH₂Cl₂/MeOH gradient (0% to 6% MeOH) to yield **3** as a yellow solid (47 mg, 0.06 mmol).

Yield 26%; mp. 106-110 °C; *R*_f = 0.61 (CH₂Cl₂/MeOH (9+1)); ¹H NMR (600 MHz, DMSO-*d*₆) δ 1.99 – 2.05 (m, 1H), 2.52 – 2.62 (m, 2H), 2.83 – 2.91 (m, 1H), 3.41 (q, *J* = 5.6 Hz, 2H), 3.58 – 3.62 (m, 6H), 3.93 (s, 2H), 4.26 (d, *J* = 6.1 Hz, 2H), 4.55 (d, *J* = 6.3 Hz, 2H), 5.03 (dd, *J* = 12.9, 5.5 Hz, 1H), 6.56 (t, *J* = 5.9 Hz, 1H), 7.02 (d, *J* = 7.1 Hz, 1H), 7.09 (d, *J* = 8.6 Hz, 1H), 7.19 (d, *J* = 8.0 Hz, 2H), 7.24 (d, *J* = 7.9 Hz, 2H), 7.51 (t, *J* = 51.4 Hz, 1H), 7.54 – 7.57 (m, 1H), 8.12 (t, *J* = 6.2 Hz, 1H), 8.68 (t, *J* = 6.4 Hz, 1H), 8.86 (s, 2H), 11.07 (s, 1H); ¹³C NMR (151 MHz, DMSO) δ 22.1, 30.9, 41.4, 41.6, 43.8, 48.5, 68.8, 69.4, 70.0, 70.3, 106.3, 106.5 (t, ¹*J* (C,F) = 238.7 Hz), 109.2, 110.6, 117.3, 127.0, 127.2, 132.0, 136.1, 137.7, 137.9, 146.3, 157.0, 157.1, 157.5 (t, ²*J* (C,F) = 29.4 Hz), 162.9, 163.1, 167.2, 168.9, 169.1, 170.0, 172.7; LC-MS (ESI) (90% H₂O + 2 mM NH₄OAc to 100% MeCN in 10 min, then 100% MeCN to 15 min, DAD 200-600 nm), *t*_R = 6.01 min, 99% purity, *m/z* [M+H]⁺ calcd for C₃₄H₃₃F₂N₉O₈ 734.2, found 734.4; HRMS (ESI) *m/z* [M+H]⁺ calcd for C₃₄H₃₃F₂N₉O₈ 734.2493, found 734.2482.



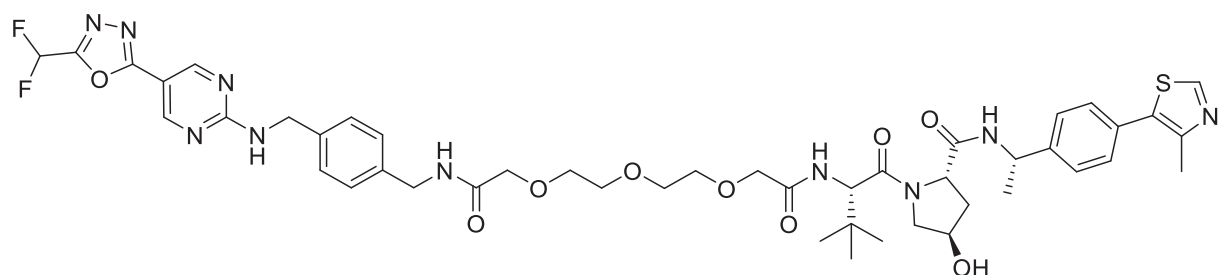
(2*S*,4*R*)-1-((*S*)-15-(*tert*-Butyl)-1-(3-(((5-(5-(difluoromethyl)-1,3,4-oxadiazol-2-yl)pyrimidin-2-yl)amino)methyl)phenyl)-3,13-dioxo-5,8,11-trioxa-2,14-diazahexadecan-16-oyl)-4-hydroxy-*N*-((*S*)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)pyrrolidine-2-carboxamide (**4**). Benzyl 3-(((5-(5-(difluoromethyl)-1,3,4-oxadiazol-2-yl)pyrimidin-2-yl)amino)methyl)benzyl)carbamate (**15**, 117 mg, 0.25 mmol) was dissolved in dry THF (10 mL). The mixture was treated with 10% m/m Pd/C under H₂ (1 atm, balloon) for 18 h. The mixture was filtered through celite and was concentrated under high vacuum. *tert*-Butyl (S)-13-((2*S*,4*R*)-4-hydroxy-2-(((*S*)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)-carbamoyl)pyrrolidine-1-carbonyl)-14,14-dimethyl-11-oxo-3,6,9-trioxa-12-azapentadecanoate (**34**, 176 mg, 0.25 mmol) was dissolved in dry CH₂Cl₂ (5 mL) and was treated with TFA (5 mL) for 2 h at room temperature. The mixture was concentrated under high vacuum. The deprotected acid compound was dissolved in dry DMF (5 mL). DIPEA (97 mg, 0.75 mmol) and HATU (105 mg, 0.28 mmol) were added under argon. The deprotected amine compound was dissolved in dry DMF (5 mL) and was added to the reaction mixture. The reaction mixture was allowed to stir at room temperature under argon for 2 h. The reaction mixture was concentrated under high vacuum. The crude product was purified by silica gel flash column chromatography using a CH₂Cl₂ /MeOH gradient (0% to 20% MeOH) to yield **4** as a white solid (117 mg, 0.12 mmol).

Yield 49%; mp. 88-92 °C; *R*_f = 0.30 (CH₂Cl₂/MeOH (9+1)); ¹H NMR (600 MHz, DMSO-*d*₆) δ 0.93 (s, 9H), 1.37 (d, *J* = 7.0 Hz, 3H), 1.75 – 1.81 (m, 1H), 2.02 – 2.08 (m, 1H), 2.45 (s, 3H), 3.54 – 3.62 (m, 10H), 3.87 – 3.97 (m, 4H), 4.27 – 4.32 (m, 3H), 4.45 (t, *J* = 8.2 Hz, 1H), 4.54 (d, *J* = 9.6 Hz, 1H), 4.59 (d, *J* = 6.3 Hz, 2H), 4.87 – 4.93 (m, 1H), 5.11 (d, *J* = 3.5 Hz, 1H), 7.12 – 7.28 (m, 4H), 7.33 – 7.39 (m, 3H), 7.41 – 7.61 (m, 3H), 8.19 (t, *J* = 6.3 Hz, 1H), 8.41 (d, *J* = 7.6 Hz, 1H), 8.72 (t, *J* = 6.4 Hz, 1H), 8.87 (s, 2H), 8.97 (s, 1H); ¹³C NMR (151 MHz, DMSO-*d*₆) δ 15.9, 22.4, 26.2, 35.7, 37.7, 41.6, 44.0, 47.7, 55.7, 56.5, 58.5, 68.7, 69.6, 69.6, 70.0, 70.3, 70.4, 106.3, 106.6 (t, ¹*J*(C,F) = 238.8 Hz), 125.5, 125.8, 125.9, 126.3, 128.2, 128.8, 129.7, 131.1, 139.2, 139.5, 144.7, 147.7, 151.4, 157.0, 157.2, 157.5 (t, ²*J*(C,F) = 29.3 Hz), 162.9, 163.1, 168.5, 169.0, 169.2, 170.4, one signal is missing due to overlapping signals; LC-MS (ESI) (90% H₂O + 2 mM NH₄OAc to 100% MeCN in 10 min, then 100% MeCN to 15 min, DAD 200-600 nm), *t*_R = 6.22 min, 96% purity, *m/z* [M+H]⁺ calcd for C₄₆H₅₆F₂N₁₀O₉S 963.4, found 963.5; HRMS (ESI) *m/z* [M+H]⁺ calcd for C₄₆H₅₆F₂N₁₀O₉S 963.3993, found 963.3990.



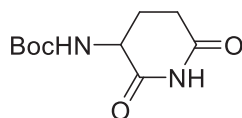
***N*¹-(3-(((5-(5-(difluoromethyl)-1,3,4-oxadiazol-2-yl)pyrimidin-2-yl)amino)methyl)benzyl)-*N*¹¹-(((*S*)-1-((2*S*,4*R*)-4-hydroxy-2-(((*S*)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)carbamoyl)pyrrolidin-1-yl)-3,3-dimethyl-1-oxobutan-2-yl)undecanediamide (5).** Benzyl (3-(((5-(5-(difluoromethyl)-1,3,4-oxadiazol-2-yl)pyrimidin-2-yl)amino)methyl)benzyl)carbamate (**15**, 70 mg, 0.15 mmol) was dissolved in dry THF (10 mL). The mixture was treated with 10% m/m Pd/C under H₂ (1 atm, balloon) for 18 h. The mixture was filtered through celite and concentrated under high vacuum. *tert*-Butyl 11-(((*S*)-1-((2*S*,4*R*)-4-hydroxy-2-(((*S*)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)carbamoyl)pyrrolidin-1-yl)-3,3-dimethyl-1-oxobutan-2-yl)amino)-11-oxoundecanoate (**35**, 105 mg, 0.15 mmol) was dissolved in dry CH₂Cl₂ (5 mL) and was treated with TFA (5 mL) for 2 h at room temperature. The mixture was concentrated under high vacuum. The deprotected acid compound was dissolved in dry DMF (5 mL). DIPEA (58 mg, 0.45 mmol) and HATU (63 mg, 0.17 mmol) were added under argon. The deprotected amine compound was dissolved in dry DMF (5 mL) and was added to the reaction mixture. The reaction mixture was allowed to stir at room temperature under argon for 2 h. The reaction mixture was concentrated under high vacuum. The crude product was purified by silica gel flash column chromatography using a CH₂Cl₂/MeOH (0% to 20% MeOH) to yield **5** as a white solid (29 mg, 0.03 mmol).

Yield 20%; *R*_f = 0.32 (CH₂Cl₂/MeOH (9+1)); ¹H NMR (500 MHz, DMSO-*d*₆) δ 0.92 (s, 9H), 1.21 (s, 10H), 1.37 (d, *J* = 7.0 Hz, 3H), 1.42 – 1.50 (m, 4H), 1.76 – 1.82 (m, 1H), 1.96 – 2.03 (m, 1H), 2.05 – 2.12 (m, 3H), 2.19 – 2.26 (m, 1H), 2.44 (s, 3H), 3.55 – 3.63 (m, 2H), 4.22 (d, *J* = 5.9 Hz, 2H), 4.25 – 4.29 (m, 1H), 4.42 (t, *J* = 8.0 Hz, 1H), 4.51 (d, *J* = 9.3 Hz, 1H), 4.58 (d, *J* = 6.3 Hz, 2H), 4.87 – 4.94 (m, 1H), 5.05 (d, *J* = 3.6 Hz, 1H), 7.10 (d, *J* = 7.4 Hz, 1H), 7.16 – 7.20 (m, 2H), 7.25 (t, *J* = 7.5 Hz, 1H), 7.35 – 7.38 (m, 2H), 7.40 – 7.62 (m, 3H), 7.72 (d, *J* = 9.3 Hz, 1H), 8.22 (t, *J* = 6.0 Hz, 1H), 8.32 (d, *J* = 7.8 Hz, 1H), 8.69 (t, *J* = 6.4 Hz, 1H), 8.86 (s, 2H), 8.96 (s, 1H); ¹³C NMR (126 MHz, DMSO-*d*₆) δ 15.9, 22.0, 22.3, 25.2, 25.4, 26.4, 28.6, 28.6, 28.7, 28.8, 34.9, 35.1, 35.3, 37.7, 41.9, 44.0, 47.6, 56.2, 56.3, 58.5, 68.7, 106.3, 106.5 (t, ¹*J*(C,F) = 238.9 Hz), 125.4, 125.6, 125.8, 126.3, 128.2, 128.8, 129.7, 131.1, 139.2, 139.9, 144.6, 147.7, 151.4, 156.9, 157.1, 157.5 (t, ²*J*(C,F) = 29.3 Hz), 162.9, 163.1, 169.6, 170.6, 172.0, 172.0; **LC-MS (ESI)** (90% H₂O + 2 mM NH₄OAc to 100% MeCN in 10 min, then 100% MeCN to 15 min, DAD 200-600 nm), *t*_R = 7.17 min, 96% purity, *m/z* [M+H]⁺ calcd for C₄₉H₆₂F₂N₁₀O₆S 957.5, found 957.7; **HRMS (ESI)** *m/z* [M+H]⁺ calcd for C₄₉H₆₂F₂N₁₀O₆S 957.4615, found 957.4615.



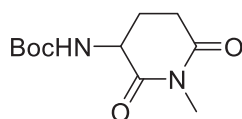
(2S,4R)-1-((S)-15-(tert-Butyl)-1-(4-(((5-(5-(difluoromethyl)-1,3,4-oxadiazol-2-yl)pyrimidin-2-yl)amino)methyl)phenyl)-3,13-dioxo-5,8,11-trioxa-2,14-diazahexadecan-16-oyl)-4-hydroxy-N-((S)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)pyrrolidine-2-carboxamide (6). Benzyl 4-(((5-(5-(difluoromethyl)-1,3,4-oxadiazol-2-yl)pyrimidin-2-yl)amino)methyl)benzyl carbamate (**16**, 93 mg, 0.2 mmol) was dissolved in dry THF (10 mL). The mixture was treated with 10% m/m Pd/C under H₂ (1 atm, balloon) for 18 h. The mixture was filtered through celite and concentrated under high vacuum. *tert*-Butyl (S)-13-((2S,4R)-4-hydroxy-2-(((S)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)carbamoyl)-pyrrolidine-1-carbonyl)-14,14-dimethyl-11-oxo-3,6,9-trioxa-12-azapentadecanoate (**34**, 141 mg, 0.2 mmol) was dissolved in dry CH₂Cl₂ (5 mL) and was treated with TFA (5 mL) for 2 h at room temperature. The mixture was concentrated under high vacuum. The deprotected acid compound was dissolved in dry DMF (5 mL). DIPEA (78 mg, 0.6 mmol) and HATU (84 mg, 0.22 mmol) were added under argon. The deprotected amine compound was dissolved in dry DMF (5 mL) and was added to the reaction mixture. The reaction mixture was allowed to stir at room temperature under argon for 2 h. The reaction mixture was concentrated under high vacuum. The crude product was purified by silica gel flash column chromatography using a CH₂Cl₂/MeOH gradient (0% to 20% MeOH) to yield **6** as a white solid (52 mg, 0.05 mmol).

Yield 27%; mp. 88-92 °C; *R*_f = 0.30 (CH₂Cl₂/MeOH (9+1)); ¹H NMR (600 MHz, DMSO-*d*₆) δ 0.93 (s, 9H), 1.37 (d, *J* = 7.0 Hz, 3H), 1.75 – 1.81 (m, 1H), 2.02 – 2.08 (m, 1H), 2.45 (s, 3H), 3.55 – 3.63 (m, 10H), 3.88 – 3.97 (m, 4H), 4.26 – 4.31 (m, 3H), 4.44 (t, *J* = 8.2 Hz, 1H), 4.54 (d, *J* = 9.5 Hz, 1H), 4.57 (d, *J* = 6.3 Hz, 2H), 4.87 – 4.94 (m, 1H), 5.11 (d, *J* = 3.5 Hz, 1H), 7.21 (d, *J* = 8.1 Hz, 2H), 7.27 (d, *J* = 8.3 Hz, 2H), 7.33 – 7.39 (m, 3H), 7.41 – 7.61 (m, 3H), 8.17 (t, *J* = 6.2 Hz, 1H), 8.41 (d, *J* = 7.7 Hz, 1H), 8.70 (t, *J* = 6.3 Hz, 1H), 8.87 (s, 2H), 8.97 (s, 1H); ¹³C NMR (151 MHz, DMSO) δ 15.9, 22.4, 26.2, 35.7, 37.7, 41.4, 43.8, 47.7, 55.7, 56.5, 58.5, 68.7, 69.5, 69.6, 69.6, 70.0, 70.3, 70.4, 106.3, 106.5 (t, ¹*J* (C,F) = 238.0 Hz), 126.3, 127.1, 127.3, 128.8, 129.7, 131.1, 137.7, 138.0, 144.7, 147.7, 151.4, 157.0, 157.1, 157.5 (t, ²*J* (C,F) = 29.3 Hz), 162.9, 163.1, 168.5, 169.0, 169.2, 170.4; **LC-MS (ESI)** (90% H₂O + 2 mM NH₄OAc to 100% MeCN in 10 min, then 100% MeCN to 15 min, DAD 200-600 nm), *t*_R = 6.20 min, 97% purity, *m/z* [M+H]⁺ calcd for C₄₆H₅₆F₂N₁₀O₉S 963.4, found 963.7; **HRMS (ESI)** *m/z* [M+H]⁺ calcd for C₄₆H₅₆F₂N₁₀O₉S 963.3993, found 963.3991.



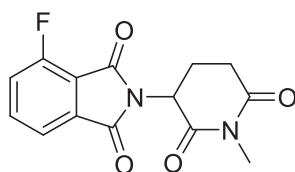
tert-Butyl (2,6-dioxopiperidin-3-yl)carbamate (42). This compound was synthesized similar to a previously reported procedure.¹⁷ Boc-Gln-OH (4.93 g, 20 mmol) was dissolved in dry THF (75 mL). 1,1'-Carbonyldiimidazole (3.89 g, 24 mmol) and DMAP (0.005 g) were added. The reaction mixture was refluxed for 10 h. The mixture was concentrated and the residue was taken up in EtOAc (300 mL). The organic layer was washed with H₂O (75 mL) and brine (75 mL). The organic layer was dried over Na₂SO₄ and loaded onto a pad of silica gel. The product was eluted with EtOAc. The solvent was evaporated to yield **42** as a white solid (3.08 g, 13.5 mmol).

Yield 68%; mp. 192-194 °C, lit.²⁴ mp. 193.7-194.4 °C; R_f = 0.31 (petroleum ether/EtOAc (1+1)); ¹H NMR (500 MHz, DMSO-*d*₆) δ 1.40 (s, 9H), 1.86 – 1.99 (m, 2H), 2.44 – 2.49 (m, 1H), 2.66 – 2.75 (m, 1H), 4.17 – 4.28 (m, 1H), 7.09 (d, J = 8.5 Hz, 1H), 10.70 (s, 1H); ¹³C NMR (126 MHz, DMSO-*d*₆) δ 24.4, 28.1, 30.9, 50.4, 78.1, 155.4, 172.4, 172.9; **LC-MS (ESI)** (90% H₂O + 2 mM NH₄OAc to 100% MeCN in 10 min, then 100% MeCN to 15 min, t_R = 3.02 min, m/z [M-H]⁻ calcd for C₁₀H₁₆N₂O₄ 227.1, found 227.0.



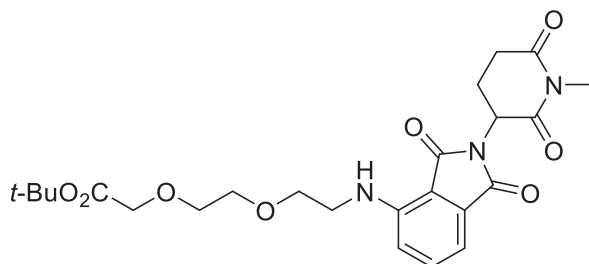
tert-Butyl (1-methyl-2,6-dioxopiperidin-3-yl)carbamate (43). This compound was synthesized similar to a previously reported procedure.¹⁷ *tert*-Butyl (2,6-dioxopiperidin-3-yl)carbamate (**42**, 2.97 g, 13 mmol) was dissolved in dry DMF (30 mL). K₂CO₃ (3.59 g, 26 mmol) and iodomethane (1.85 g, 13 mmol) were added. The reaction mixture was sonicated for 2 h. The mixture was concentrated under high vacuum. The residue was taken up in EtOAc (15 mL). The organic layer was washed with 1 N NaOH (2 × 40 mL), H₂O (40 mL) and brine (40 mL). The organic layer was dried over Na₂SO₄, filtered and concentrated. The crude product was purified by silica gel column chromatography using petroleum ether/EtOAc (2+1) as eluent to yield **43** as a white solid (1.55 g, 6.40 mmol).

Yield 49%; mp. 86-88 °C, lit.¹⁷ mp. 84-86 °C; R_f = 0.41 (petroleum ether/EtOAc (2+1)); ¹H NMR (500 MHz, DMSO-*d*₆) δ 1.40 (s, 9H), 1.85 – 2.00 (m, 2H), 2.60 – 2.68 (m, 1H), 2.75 – 2.84 (m, 1H), 2.97 (s, 3H), 4.22 – 4.37 (m, 1H), 7.16 (d, J = 8.6 Hz, 1H); ¹³C NMR (126 MHz, DMSO) δ 23.5, 26.4, 28.1, 31.1, 50.9, 78.1, 155.4, 172.0, 172.2; **LC-MS (ESI)** (90% H₂O + 2 mM NH₄OAc to 100% MeCN in 10 min, then 100% MeCN to 15 min), t_R = 4.05 min, m/z [M-H]⁻ calcd for C₁₁H₁₈N₂O₄ 241.1, found 241.1.



4-Fluoro-2-(1-methyl-2,6-dioxopiperidin-3-yl)isoindoline-1,3-dione (44). This compound was synthesized similar to a previously reported procedure.¹⁷ 3-Fluorophthalic anhydride (1.28 g, 9 mmol) and *tert*-butyl (1-methyl-2,6-dioxopiperidin-3-yl)carbamate (**43**, 1.45 g, 6 mmol) were put in a flask. A solution of sodium acetate (995 mg, 7.2 mmol) in glacial acetate (20 mL) was added and the reaction mixture was refluxed for 6 h. The reaction mixture was allowed to cool to room temperature and was then poured onto H₂O (100 mL). The precipitate was collected, washed with H₂O (3 × 5 mL) and petroleum ether (3 × 5 mL) and it was dried under high vacuum to yield **44** as a light purple solid (1.30 g, 4.48 mmol).

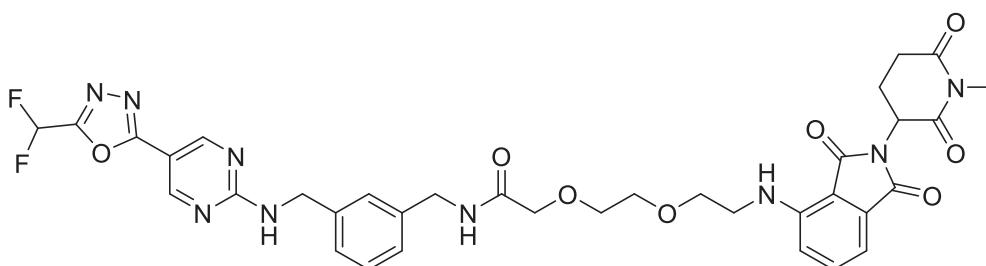
Yield 75%; mp. 200-202 °C, lit.¹⁷ mp. 196-198 °C; *R*_f = 0.23 (petroleum ether/EtOAc (2+1)); ¹H NMR (500 MHz, DMSO-*d*₆) δ 2.05 – 2.12 (m, 1H), 2.52 – 2.59 (m, 1H), 2.74 – 2.81 (m, 1H), 2.91 – 3.00 (m, 1H), 3.03 (s, 3H), 5.22 (dd, *J* = 13.1, 5.4 Hz, 1H), 7.74 (t, *J* = 8.9 Hz, 1H), 7.79 (d, *J* = 7.3 Hz, 1H), 7.92 – 7.98 (m, 1H); ¹³C NMR (126 MHz, DMSO-*d*₆) δ 21.0, 26.6, 31.0, 49.6, 117.0 (d, ²*J*(C, F) = 12.6 Hz), 120.0 (d, ⁴*J*(C,F) = 3.0 Hz), 123.0 (d, ²*J*(C, F) = 19.6 Hz), 133.4, 138.0 (d, ³*J*(C,F) = 7.7 Hz), 156.8 (d, ¹*J*(C,F) = 262.4 Hz), 163.9, 166.0 (d, ³*J*(C,F) = 2.9 Hz), 169.3, 171.6; LC-MS (ESI) (90% H₂O + 2 mM NH₄OAc to 100% MeCN in 10 min, then 100% MeCN to 15 min, DAD 220-600 nm), *t*_R = 4.51 min, 100% purity, *m/z* [M+H]⁺ calcd for C₁₄H₁₁FN₂O₄ 291.1, found 291.0.



***tert*-Butyl 2-(2-(2-((2-(1-methyl-2,6-dioxopiperidin-3-yl)-1,3-dioxoisindolin-4-yl)amino)ethoxy)ethoxy)acetate (45).** The orthogonally protected linker *tert*-butyl 3-oxo-1-phenyl-2,7,10-trioxa-4-azadodecan-12-oate (**19**, 1.06 g, 3 mmol) was dissolved in dry EtOAc (30 mL) and treated with 10% m/m Pd/C. The reaction mixture was stirred under H₂ (1 atm, balloon) for 18 h. The mixture was filtered through celite and the filtrate was concentrated. The residue was redissolved in dry DMSO (30 mL) and DIPEA (0.776 g, 6 mmol) and 4-fluoro-2-(1-methyl-2,6-dioxopiperidin-3-yl)isoindoline-1,3-dione (**44**, 871 mg, 3 mmol) were added. The reaction mixture was stirred at 90 °C for 18 h. The reaction mixture was allowed to cool to room temperature. Then it was poured onto half-saturated brine (300 mL) and it was extracted with EtOAc (2 × 150 mL). The combined organic layers were washed with 5% aqueous LiCl solution (150 mL) and brine (150 mL), dried over Na₂SO₄, filtered and concentrated. The crude

product was purified by silica gel column chromatography using a gradient from petroleum ether/EtOAc (1+1) to petroleum ether/EtOAc (1+2) to yield **45** as a yellowish-green resin (665 mg, 1.36 mmol).

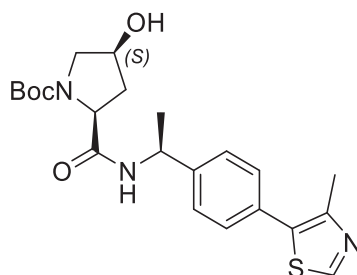
Yield 45%; R_f = 0.33 (petroleum ether/EtOAc (1+1)); $^1\text{H NMR}$ (500 MHz, DMSO- d_6) δ 1.41 (s, 9H), 2.01 – 2.08 (m, 1H), 2.51 – 2.59 (m, 1H), 2.72 – 2.79 (m, 1H), 2.90 – 2.99 (m, 1H), 3.02 (s, 3H), 3.47 (q, J = 5.6 Hz, 2H), 3.57 – 3.60 (m, 4H), 3.63 (t, J = 5.4 Hz, 2H), 3.97 (s, 2H), 5.12 (dd, J = 13.0, 5.4 Hz, 1H), 6.60 (t, J = 5.9 Hz, 1H), 7.04 (d, J = 7.0 Hz, 1H), 7.15 (d, J = 8.5 Hz, 1H), 7.59 (dd, J = 8.5, 7.1 Hz, 1H); $^{13}\text{C NMR}$ (126 MHz, DMSO- d_6) δ 21.3, 26.5, 27.7, 31.1, 41.7, 49.1, 68.2, 68.8, 69.6, 69.9, 80.6, 109.2, 110.6, 117.4, 132.0, 136.2, 146.4, 167.2, 168.9, 169.3, 169.7, 171.7; **LC-MS (ESI)** (90% H_2O + 2 mM NH_4OAc to 100% MeCN in 10 min, then 100% MeCN to 15 min, DAD 220-600 nm), t_R = 6.83 min, 99% purity, m/z $[\text{M-H}]^-$ calcd for $\text{C}_{24}\text{H}_{31}\text{N}_3\text{O}_8$ 488.2, found 488.2.



***N*-(3-(((5-(5-(difluoromethyl)-1,3,4-oxadiazol-2-yl)pyrimidin-2-yl)amino)methyl)benzyl)-2-(2-(2-((2-(1-methyl-2,6-dioxopiperidin-3-yl)-1,3-dioxoisindolin-4-yl)amino)ethoxy)ethoxy)acetamide (1(-)).** Benzyl (3-(((5-(5-(difluoromethyl)-1,3,4-oxadiazol-2-yl)pyrimidin-2-yl)amino)methyl)benzyl)-carbamate (**15**, 93 mg, 0.2 mmol) was dissolved in dry THF (10 mL) and was treated with 10% m/m Pd/C under H_2 (1 atm, balloon) for 18 h. The mixture was filtered through celite and concentrated under high vacuum. *tert*-Butyl 2-(2-(2-((2-(1-methyl-2,6-dioxopiperidin-3-yl)-1,3-dioxoisindolin-4-yl)amino)ethoxy)ethoxy)acetate (**45**, 98 mg, 0.2 mmol) was dissolved in dry CH_2Cl_2 (5 mL) and was treated with TFA (5 mL) for 2 h at room temperature. The mixture was concentrated under high vacuum. The deprotected acid compound was dissolved in dry DMF (5 mL). DIPEA (78 mg, 0.6 mmol) and HATU (84 mg, 0.22 mmol) were added under argon. The deprotected amine compound was dissolved in dry DMF (5 mL) and was added. The reaction mixture was allowed to stir at room temperature under argon for 18 h. The mixture was concentrated under high vacuum. The crude product was purified by silica gel flash column chromatography using a $\text{CH}_2\text{Cl}_2/\text{MeOH}$ gradient (0% to 4% MeOH) to yield **1(-)** as a yellow solid (72 mg, 0.096 mmol).

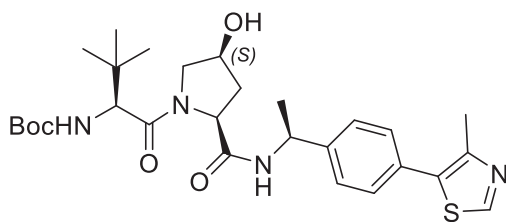
Yield 48%; mp. 82-88 °C; R_f = 0.24 ($\text{CH}_2\text{Cl}_2/\text{MeOH}$ (19+1)); $^1\text{H NMR}$ (600 MHz, DMSO- d_6) δ 2.00 – 2.06 (m, 1H), 2.51 – 2.57 (m, 1H), 2.72 – 2.77 (m, 1H), 2.88 – 2.96 (m, 1H), 3.01 (s, 3H), 3.41 (q, J = 5.6 Hz, 2H), 3.58 – 3.61 (m, 6H), 3.92 (s, 2H), 4.28 (d, J = 6.2 Hz, 2H), 4.57 (d, J = 6.3 Hz, 2H), 5.10 (dd, J = 13.0, 5.4 Hz, 1H), 6.56 (t, J = 5.9 Hz, 1H), 7.02 (d, J = 7.0 Hz, 1H), 7.08 – 7.13 (m, 2H), 7.16 – 7.26 (m, 3H), 7.51 (t, J = 5.1 Hz, 1H), 7.56 (dd, J = 8.6, 7.1 Hz, 1H), 8.15 (t, J = 6.2 Hz, 1H), 8.70 (t, J = 6.4 Hz, 1H), 8.86 (s, 2H); $^{13}\text{C NMR}$ (151 MHz, DMSO- d_6) δ 21.3, 26.6, 31.1, 41.6, 41.6, 44.0, 49.1, 68.8, 69.4, 70.0, 70.3, 106.3, 106.5 (t, $^1J(\text{C},\text{F})$ = 238.3 Hz), 109.2, 110.7, 117.4, 125.5, 125.7,

125.9, 128.2, 132.0, 136.2, 139.2, 139.5, 146.3, 157.0, 157.1, 157.5 ($t, {}^2J(\text{C},\text{F}) = 29.3 \text{ Hz}$), 162.9, 163.1, 167.2, 168.9, 169.2, 169.8, 171.7; **LC-MS (ESI)** (90% H_2O + 2 mM NH_4OAc to 100% MeCN in 10 min, then 100% MeCN to 15 min, DAD 220-600 nm), $t_{\text{R}} = 6.34 \text{ min}$, 99% purity, m/z $[\text{M}+\text{H}]^+$ calcd for $\text{C}_{35}\text{H}_{35}\text{F}_2\text{N}_9\text{O}_8$ 748.3, found 748.5; **HRMS (ESI)** m/z $[\text{M}+\text{H}]^+$ calcd for $\text{C}_{35}\text{H}_{35}\text{F}_2\text{N}_9\text{O}_8$ 748.2649, found 748.2649.



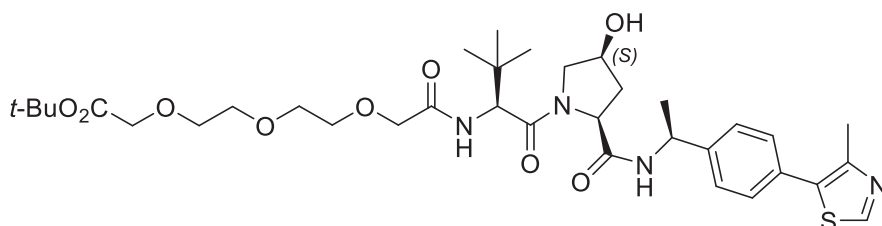
tert-Butyl (2*S*,4*S*)-4-hydroxy-2-(((*S*)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)carbamoyl)-pyrrolidine-1-carboxylate (46). *tert*-Butyl (*S*)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl carbamate (**38**, 1.27 g, 4 mmol) was dissolved in dry CH_2Cl_2 (5 mL) and was treated with TFA (5 mL) for 2 h at room temperature. The mixture was concentrated under high vacuum. (2*S*,4*S*)-1-(*tert*-Butoxycarbonyl)-4-hydroxypyrrolidine-2-carboxylic acid (925 mg, 4 mmol) was dissolved in dry DMF (10 mL). DIPEA (1.81 g, 14 mmol) and HATU (1.67 g, 4.4 mmol) were added under argon. The deprotected amine compound was dissolved in dry DMF (10 mL) and was added to the reaction mixture. The reaction mixture was stirred under argon for 4 h. The reaction mixture was concentrated under high vacuum. The residue was diluted with water (50 mL) and was extracted with EtOAc (3 $\times$ 50 mL). The combined organic layers were washed with saturated NaHCO_3 solution (50 mL) and brine (50 mL). The organic phase was dried over Na_2SO_4 , filtered and concentrated. The crude product was purified by silica gel column chromatography using $\text{CH}_2\text{Cl}_2/\text{MeOH}$ (19+1) as eluent to yield **46** as a white solid (449 mg, 1.04 mmol).

Yield 26%; mp. 62-64 $^\circ\text{C}$; $R_{\text{f}} = 0.28$ ($\text{CH}_2\text{Cl}_2/\text{MeOH}$ (19+1)); **^1H NMR** (600 MHz, $\text{DMSO}-d_6$) δ 1.32 (s, 6H), 1.37 – 1.43 (m, 6H), 1.64 – 1.70 (m, 1H), 2.28 – 2.37 (m, 1H), 2.45 (s, 3H), 3.15 – 3.24 (m, 1H), 3.46 – 3.50 (m, 1H), 4.10 – 4.18 (m, 2H), 4.93 – 5.03 (m, 1H), 5.14 – 5.22 (m, 1H), 7.38 – 7.47 (m, 4H), 8.30 and 8.39 (each d, $J = 7.5 \text{ Hz}$, $J = 7.8 \text{ Hz}$, 1H, major and minor rotamer), 8.98 (s, 1H); **^{13}C NMR** (151 MHz, $\text{DMSO}-d_6$) δ 15.9, 21.8, 27.9, 38.6, 47.5, 54.4, 58.8, 68.0, 78.7, 126.5, 128.8, 129.9, 131.0, 144.1, 147.8, 151.5, 153.3, 172.1; **LC-MS (ESI)** (90% H_2O + 2 mM NH_4OAc to 100% MeCN in 10 min, then 100% MeCN to 15 min, DAD 220-600 nm), $t_{\text{R}} = 5.69 \text{ min}$, 96% purity, m/z $[\text{M}-\text{H}]^-$ calcd for $\text{C}_{22}\text{H}_{29}\text{N}_3\text{O}_4\text{S}$ 430.2, found 430.2.



tert-Butyl ((S)-1-((2S,4S)-4-hydroxy-2-(((S)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)carbamoyl)pyrrolidin-1-yl)-3,3-dimethyl-1-oxobutan-2-yl)carbamate (47). *tert*-Butyl (2S,4S)-4-hydroxy-2-(((S)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)carbamoyl)pyrrolidine-1-carboxylate (**46**, 432 mg, 1 mmol) was dissolved in dry CH₂Cl₂ (5 mL) and was treated with TFA (5 mL) for 2 h. The mixture was concentrated under high vacuum. Boc-Tle-OH (**39**, 231 mg, 1 mmol) was dissolved in dry DMF (5 mL). HATU (418 mg, 1.1 mmol) and DIPEA (452 mg, 3.5 mmol) were added under argon. The deprotected amine compound was dissolved in dry DMF (5 mL) and was added to the reaction mixture. The reaction mixture was stirred for 18 h under argon. The mixture was concentrated under high vacuum. The crude product was purified by silica gel column chromatography using EtOAc as eluent to yield **47** as a white solid (339 mg, 0.62 mmol).

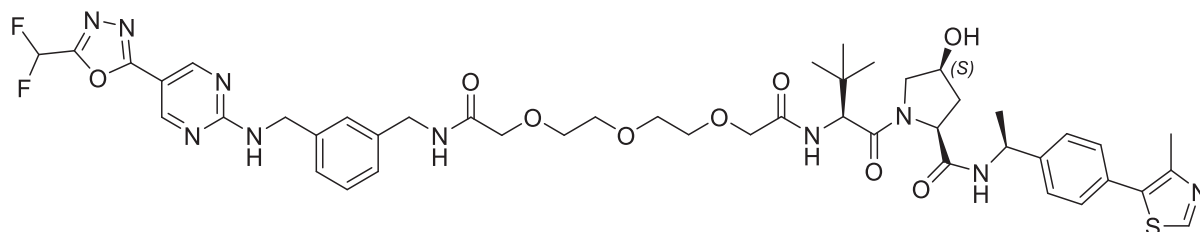
Yield 62%; mp. 100-102 °C; *R*_f = 0.24 (CH₂Cl₂ + MeOH (19+1)); ¹H NMR (500 MHz, DMSO-*d*₆) δ 0.95 (s, 9H), 1.36 – 1.40 (m, 12H), 1.62 – 1.68 (m, 1H), 2.29 – 2.36 (m, 1H), 2.46 (s, 3H), 3.38 (dd, *J* = 10.1, 5.3 Hz, 1H), 3.81 – 3.90 (m, 1H), 4.10 (d, *J* = 8.7 Hz, 1H), 4.17 – 4.24 (m, 1H), 4.36 (dd, *J* = 8.8, 6.0 Hz, 1H), 4.88 – 4.96 (m, 1H), 5.31 (d, *J* = 6.7 Hz, 1H), 6.51 (d, *J* = 8.8 Hz, 1H), 7.37 – 7.40 (m, 2H), 7.42 – 7.45 (m, 2H), 8.33 (d, *J* = 7.6 Hz, 1H), 8.98 (s, 1H); ¹³C NMR (126 MHz, DMSO-*d*₆) δ 15.9, 22.2, 26.3, 28.1, 34.7, 36.7, 47.8, 55.4, 58.4, 58.6, 69.0, 78.1, 126.3, 128.8, 129.7, 131.0, 144.3, 147.7, 151.4, 155.5, 170.1, 171.0; LC-MS (ESI) (90% H₂O + 2 mM NH₄OAc to 100% MeCN in 10 min, then 100% MeCN to 15 min, DAD 220-600 nm), *t*_R = 6.55 min, 95% purity, *m/z* [M+H]⁺ calcd for C₂₈H₄₀N₄O₅S 545.3, found 545.5.



tert-Butyl (S)-13-((2S,4S)-4-hydroxy-2-(((S)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)carbamoyl)pyrrolidine-1-carbonyl)-14,14-dimethyl-11-oxo-3,6,9-trioxa-12-azapentadecanoate (48). *tert*-Butyl ((S)-1-((2S,4S)-4-hydroxy-2-(((S)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)carbamoyl)pyrrolidin-1-yl)-3,3-dimethyl-1-oxobutan-2-yl)carbamate (**47**, 327 mg, 0.6 mmol) was dissolved in dry CH₂Cl₂ (5 mL) and was treated with TFA (5 mL). The mixture was stirred for 2 h at room temperature. The mixture was concentrated under high vacuum. 13,13-Dimethyl-11-oxo-3,6,9,12-tetraoxatetradecanoic acid (**31**, 167 mg, 0.6 mmol) was dissolved in dry DMF (5 mL). HATU (251 mg, 0.66 mmol) and DIPEA (271 mg, 2.1 mmol) were added under argon. The deprotected (-)VHL-ligand was dissolved in dry DMF (5

mL) and was added to the mixture, containing the activated acid compound. The reaction mixture was stirred at room temperature under argon for 18 h. The mixture was concentrated under high vacuum. The crude product was purified by silica gel column chromatography using CH₂Cl₂/MeOH (19+1) as eluent to yield **48** as a white solid (267 mg, 0.38 mmol).

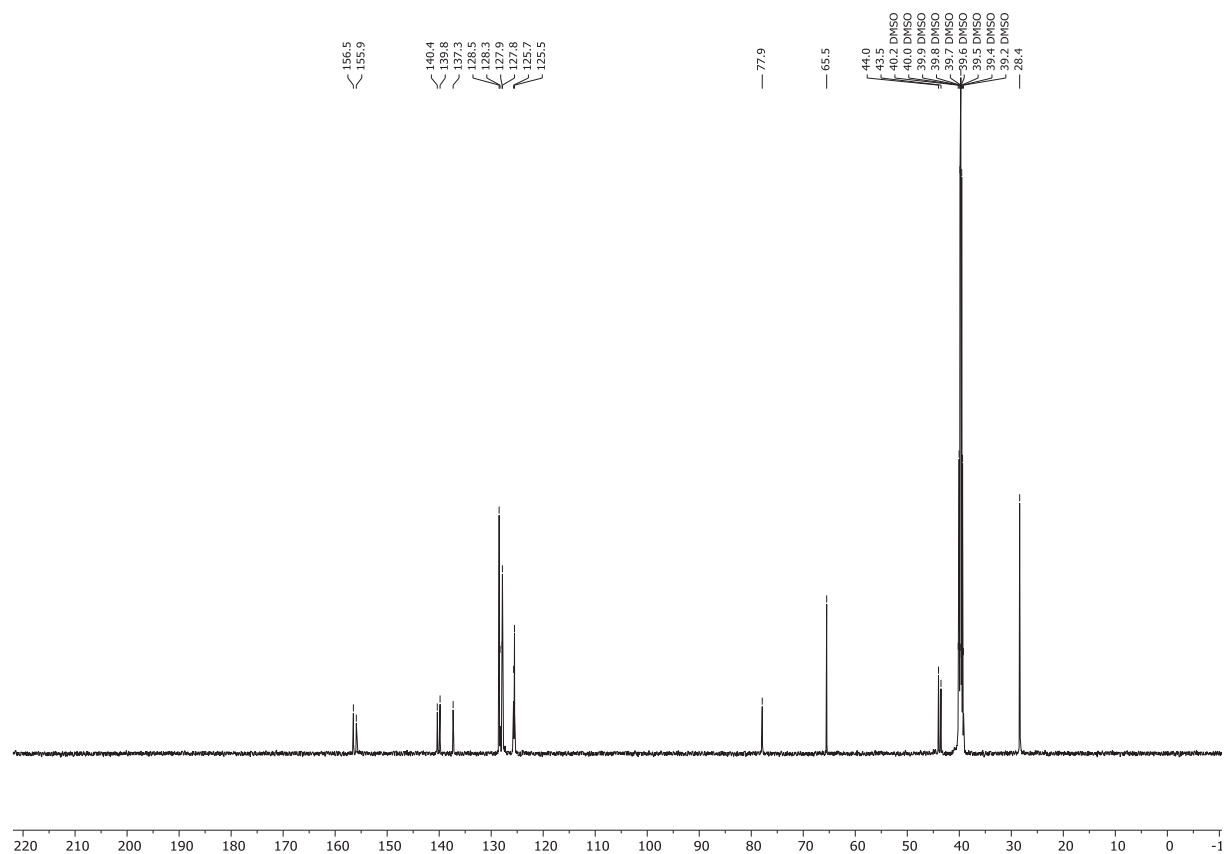
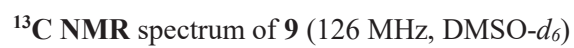
Yield 63%; *R*_f = 0.18 (CH₂Cl₂/MeOH (19+1)); ¹H NMR (600 MHz, DMSO-*d*₆) δ 0.95 (s, 9H), 1.38 (d, *J* = 7.0 Hz, 3H), 1.42 (s, 9H), 1.62 – 1.67 (m, 1H), 2.30 – 2.36 (m, 1H), 2.46 (s, 3H), 3.40 (dd, *J* = 10.1, 5.4 Hz, 1H), 3.55 – 3.62 (m, 8H), 3.86 (dd, *J* = 10.1, 5.7 Hz, 1H), 3.95 (d, *J* = 4.2 Hz, 2H), 3.97 (s, 2H), 4.18 – 4.24 (m, 1H), 4.35 (dd, *J* = 8.7, 6.3 Hz, 1H), 4.50 (d, *J* = 9.2 Hz, 1H), 4.90 – 4.96 (m, 1H), 5.32 (d, *J* = 6.7 Hz, 1H), 7.35 – 7.40 (m, 3H), 7.42 – 7.46 (m, 2H), 8.37 (d, *J* = 7.7 Hz, 1H), 8.98 (s, 1H); ¹³C NMR (151 MHz, DMSO) δ 15.9, 22.2, 26.2, 27.7, 35.1, 36.8, 47.8, 55.5, 55.8, 58.5, 68.1, 68.9, 69.5, 69.7, 69.9, 70.4, 80.6, 126.4, 128.8, 129.8, 131.1, 144.3, 147.8, 151.4, 168.8, 169.3, 169.3, 170.9, one signal is missing due to overlapping signals; LC-MS (ESI) (90% H₂O + 2 mM NH₄OAc to 100% MeCN in 10 min, then 100% MeCN to 15 min, DAD 220-600 nm), *t*_R = 6.55 min, 99% purity, *m/z* [M+H]⁺ calcd for C₃₅H₅₂N₄O₉S 705.4, found 705.6.



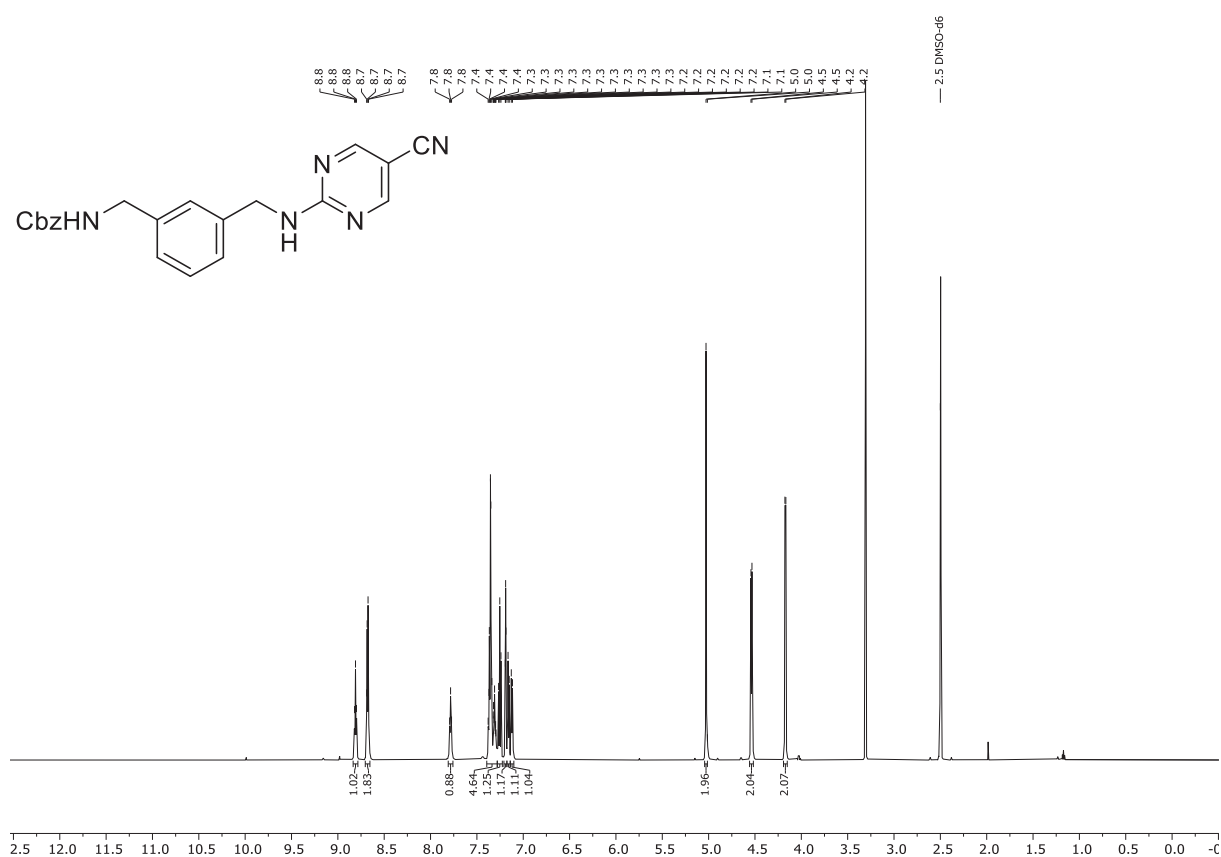
(2S,4S)-1-((S)-15-(*tert*-Butyl)-1-(3-(((5-(5-(difluoromethyl)-1,3,4-oxadiazol-2-yl)pyrimidin-2-yl)amino)methyl)phenyl)-3,13-dioxo-5,8,11-trioxa-2,14-diazahexadecan-16-oyl)-4-hydroxy-N-((S)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)pyrrolidine-2-carboxamide (4(-)). Benzyl (3-(((5-(5-(difluoromethyl)-1,3,4-oxadiazol-2-yl)pyrimidin-2-yl)amino)methyl)benzyl)carbamate (**15**, 70 mg, 0.15 mmol) was dissolved in dry THF (10 mL). The mixture was treated with 10% m/m Pd/C under H₂ (1 atm, balloon) for 18 h. The mixture was filtered through celite and concentrated under high vacuum. *tert*-Butyl (S)-13-((2S,4S)-4-hydroxy-2-(((S)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)carbamoyl)-pyrrolidine-1-carbonyl)-14,14-dimethyl-11-oxo-3,6,9-trioxa-12-azapentadecanoate (**48**, 106 mg, 0.15 mmol) was dissolved in dry CH₂Cl₂ (5 mL) and was treated with TFA (5 mL) for 2 h at room temperature. The mixture was concentrated under high vacuum. The deprotected acid compound was dissolved in dry DMF (5 mL). DIPEA (58 mg, 0.45 mmol) and HATU (63 mg, 0.165 mmol) were added under argon. The deprotected amine compound was dissolved in dry DMF (5 mL) and was added to the reaction mixture. The reaction mixture was allowed to stir at room temperature under argon for 18 h. The reaction mixture was concentrated under high vacuum. The crude product was purified by silica gel flash column chromatography using a CH₂Cl₂/MeOH gradient (0% to 20% MeOH) to yield **4(-)** as a white solid (71 mg, 0.074 mmol).

Yield 49%; mp. 80-86 °C; R_f = 0.49 (CH₂Cl₂/MeOH (9+1)); **¹H NMR** (600 MHz, DMSO-*d*₆) δ 0.94 (s, 9H), 1.37 (d, J = 6.9 Hz, 3H), 1.61 – 1.67 (m, 1H), 2.28 – 2.35 (m, 1H), 2.45 (s, 3H), 3.39 (dd, J = 10.1, 5.4 Hz, 1H), 3.53 – 3.62 (m, 8H), 3.85 (dd, J = 10.2, 5.7 Hz, 1H), 3.88 – 3.96 (m, 4H), 4.17 – 4.23 (m, 1H), 4.29 (d, J = 6.2 Hz, 2H), 4.34 (dd, J = 8.7, 6.3 Hz, 1H), 4.50 (d, J = 9.3 Hz, 1H), 4.59 (d, J = 6.4 Hz, 2H), 4.89 – 4.95 (m, 1H), 5.31 (d, J = 6.7 Hz, 1H), 7.11 – 7.28 (m, 4H), 7.35 – 7.40 (m, 3H), 7.42 – 7.61 (m, 3H), 8.19 (t, J = 6.3 Hz, 1H), 8.37 (d, J = 7.6 Hz, 1H), 8.72 (t, J = 6.4 Hz, 1H), 8.87 (s, 2H), 8.97 (s, 1H); **¹³C NMR** (151 MHz, DMSO-*d*₆) δ 15.9, 22.2, 26.2, 35.2, 36.8, 41.6, 44.0, 47.8, 55.5, 55.8, 58.5, 68.9, 69.5, 69.6, 70.0, 70.3, 70.3, 106.3, 106.6 (t, ^1J (C,F) = 238.3 Hz), 125.5, 125.8, 125.9, 126.4, 128.2, 128.8, 129.8, 131.0, 139.2, 139.5, 144.3, 147.8, 151.4, 157.0, 157.2, 157.5 (t, ^2J (C,F) = 29.3 Hz), 162.9, 163.1, 168.8, 169.2, 169.3, 170.9, one signal is missing due to overlapping signals; **LC-MS (ESI)** (90% H₂O + 2 mM NH₄OAc to 100% MeCN in 10 min, then 100% MeCN to 15 min, DAD 220-600 nm), t_R = 6.34 min, 97% purity, m/z [M+H]⁺ calcd for C₄₆H₅₆F₂N₁₀O₉S 963.4, found 963.7; **HRMS (ESI)** m/z [M+H]⁺ calcd for C₄₆H₅₆F₂N₁₀O₉S 963.3993, found 963.3991.

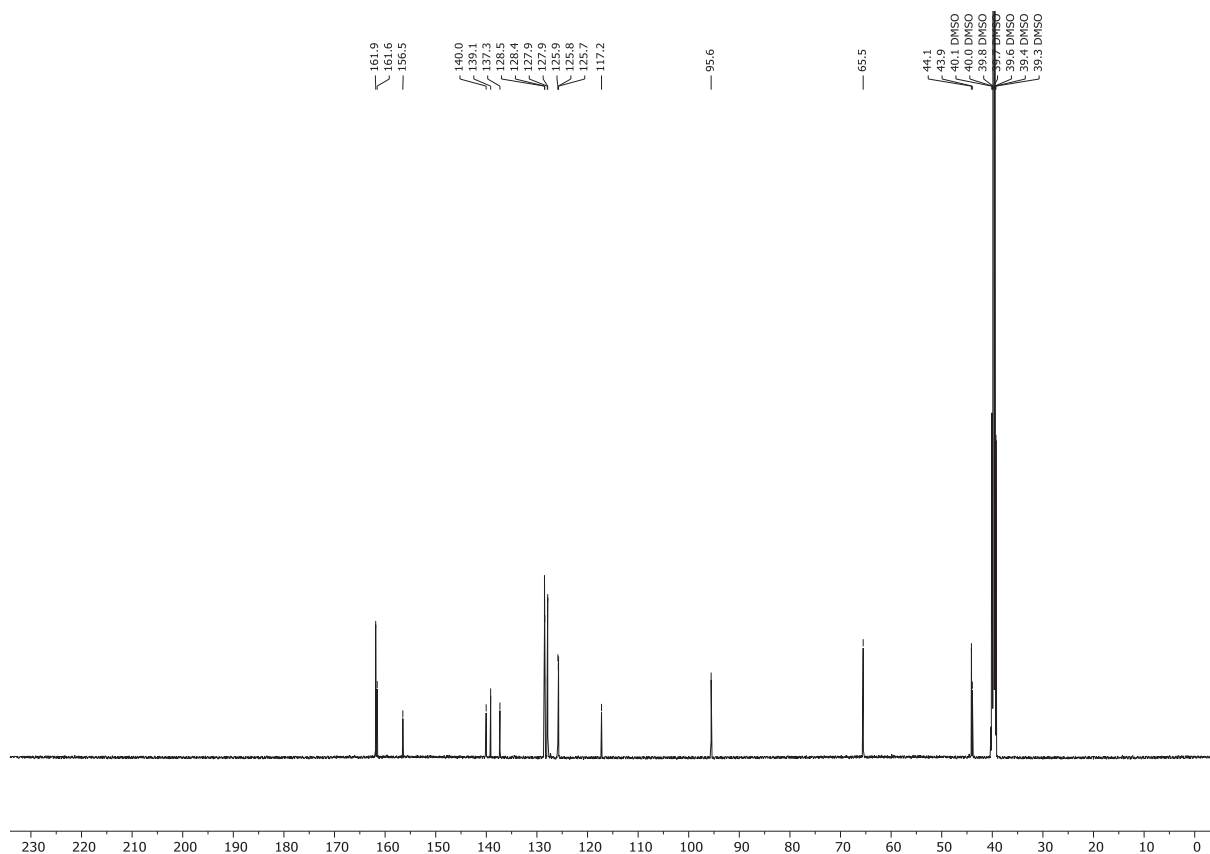
¹H NMR spectrum of **9** (600 MHz, DMSO-*d*₆)



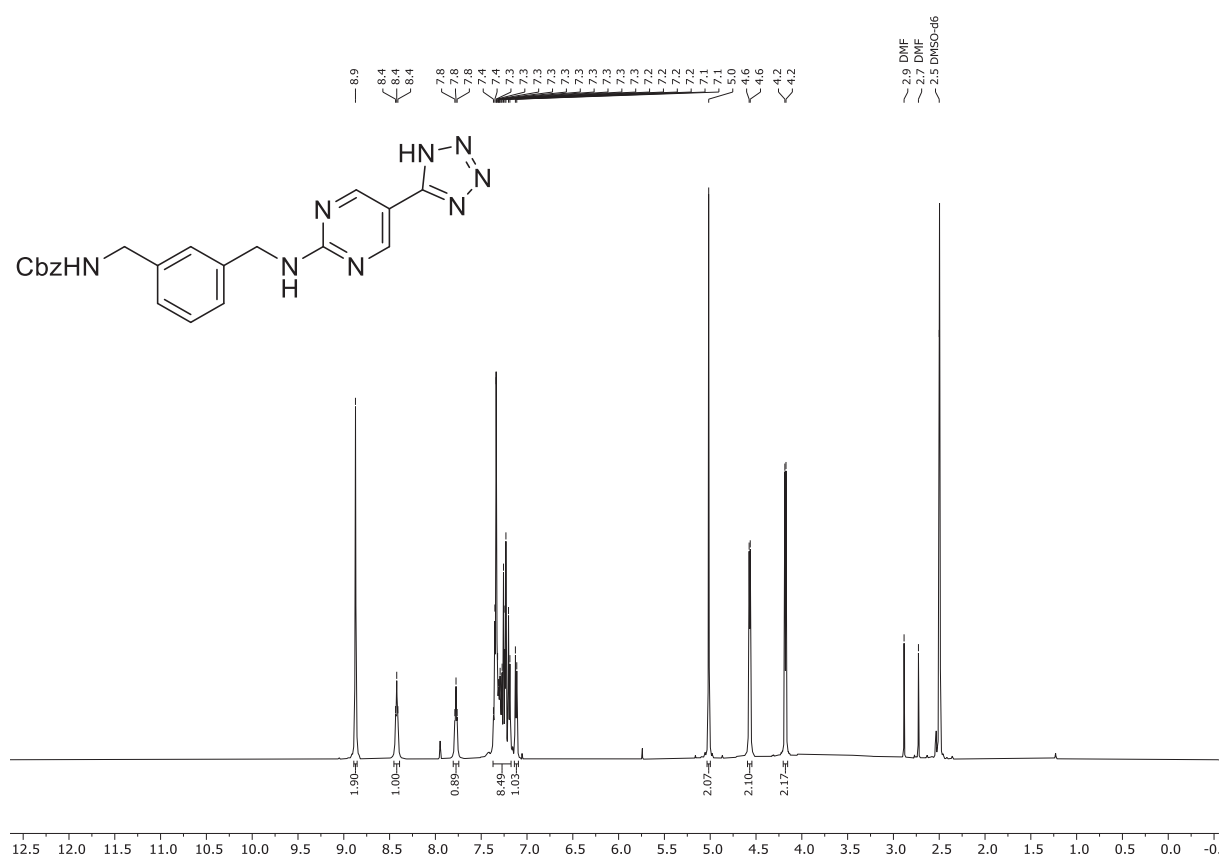
¹H NMR spectrum of **11** (600 MHz, DMSO-*d*₆)



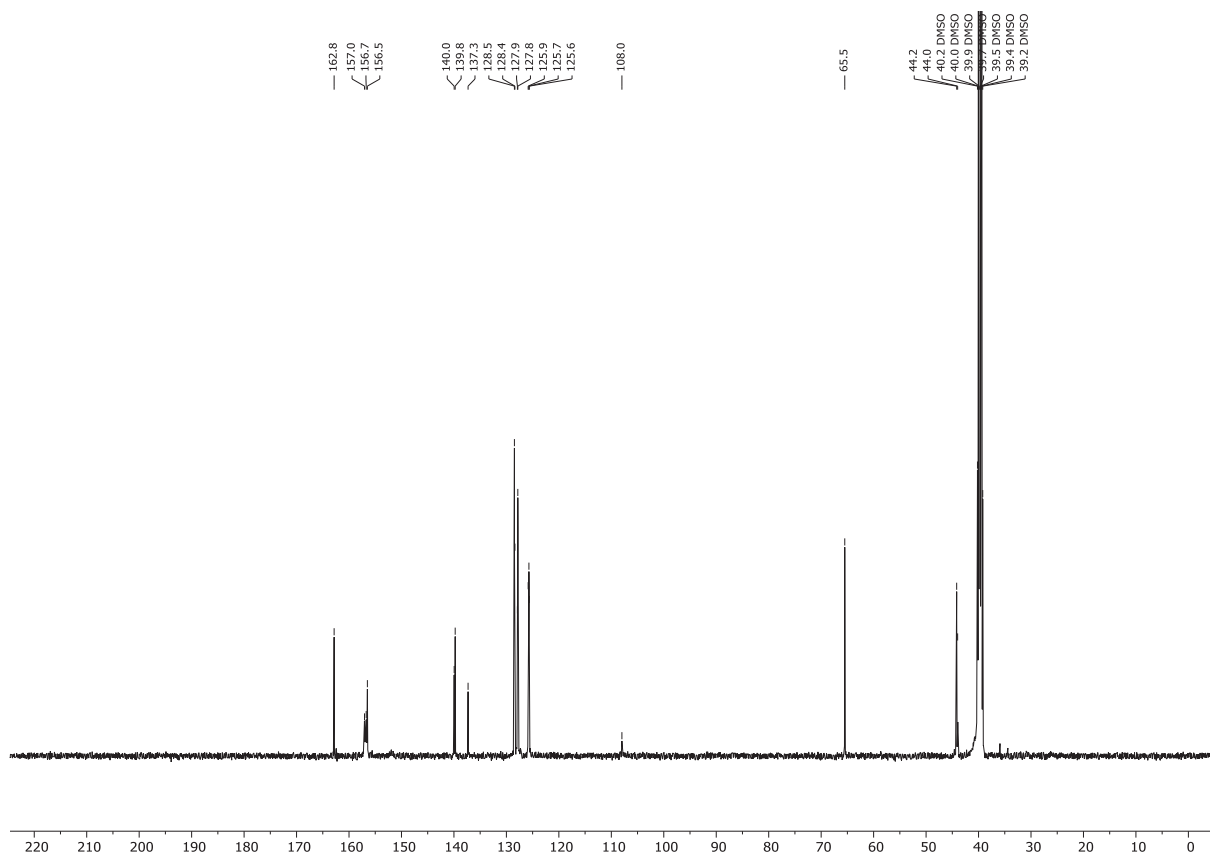
¹³C NMR spectrum of **11** (151 MHz, DMSO-*d*₆)



¹H NMR spectrum of 13 (500 MHz, DMSO-*d*₆)



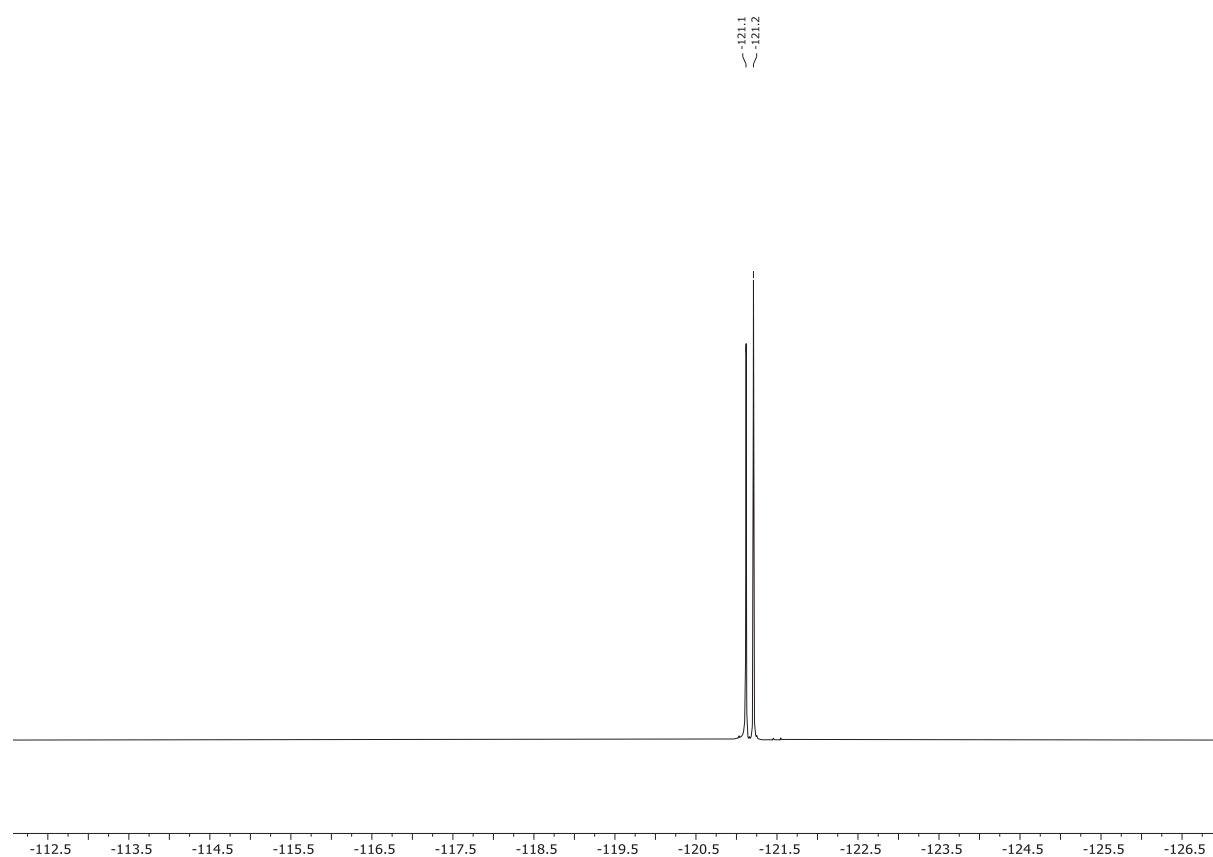
¹³C NMR spectrum of 13 (126 MHz, DMSO-*d*₆)



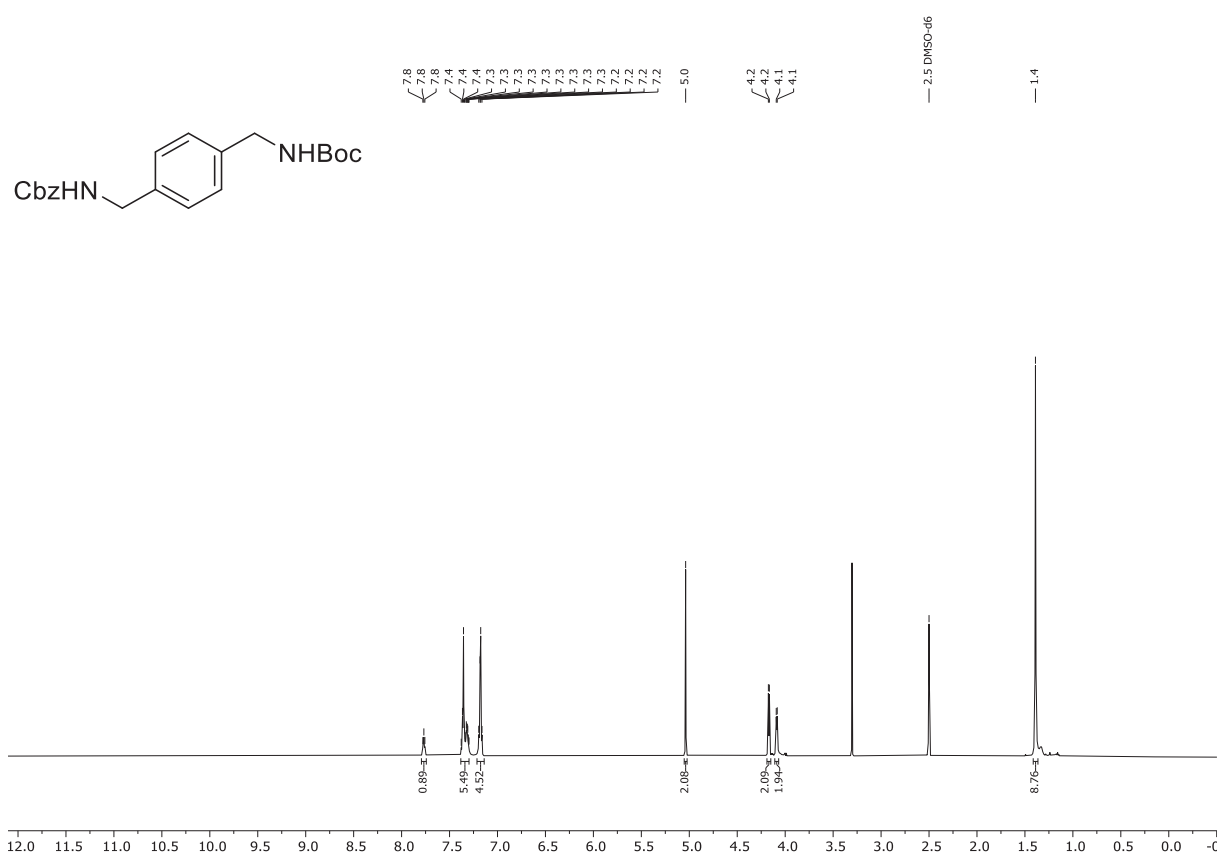
[illegible]

13C NMR spectrum of compound 10 in DMSO-d₆. The x-axis represents chemical shift in ppm, ranging from 230 to 0. The spectrum shows several sharp peaks. Key peaks are labeled with their chemical shifts: 163.3, 162.1, 157.9, 157.7, 157.5, 157.3, 157.2, 156.5, 140.0, 139.4, 137.3, 128.5, 128.4, 127.9, 127.8, 125.9, 125.8, 108.3, 106.7, 106.5, 105.2, 65.5, 44.2, 44.0, 43.8, 40.0, 39.8, 39.7, 39.6, 39.4, and 39.3 ppm. A cluster of peaks between 156 and 164 ppm is associated with the pyridine ring, peaks between 105 and 140 ppm with the furan ring, and the peak at 65.5 ppm with the methoxy group. The cluster of peaks between 39.3 and 44.2 ppm is associated with the sugar moiety.

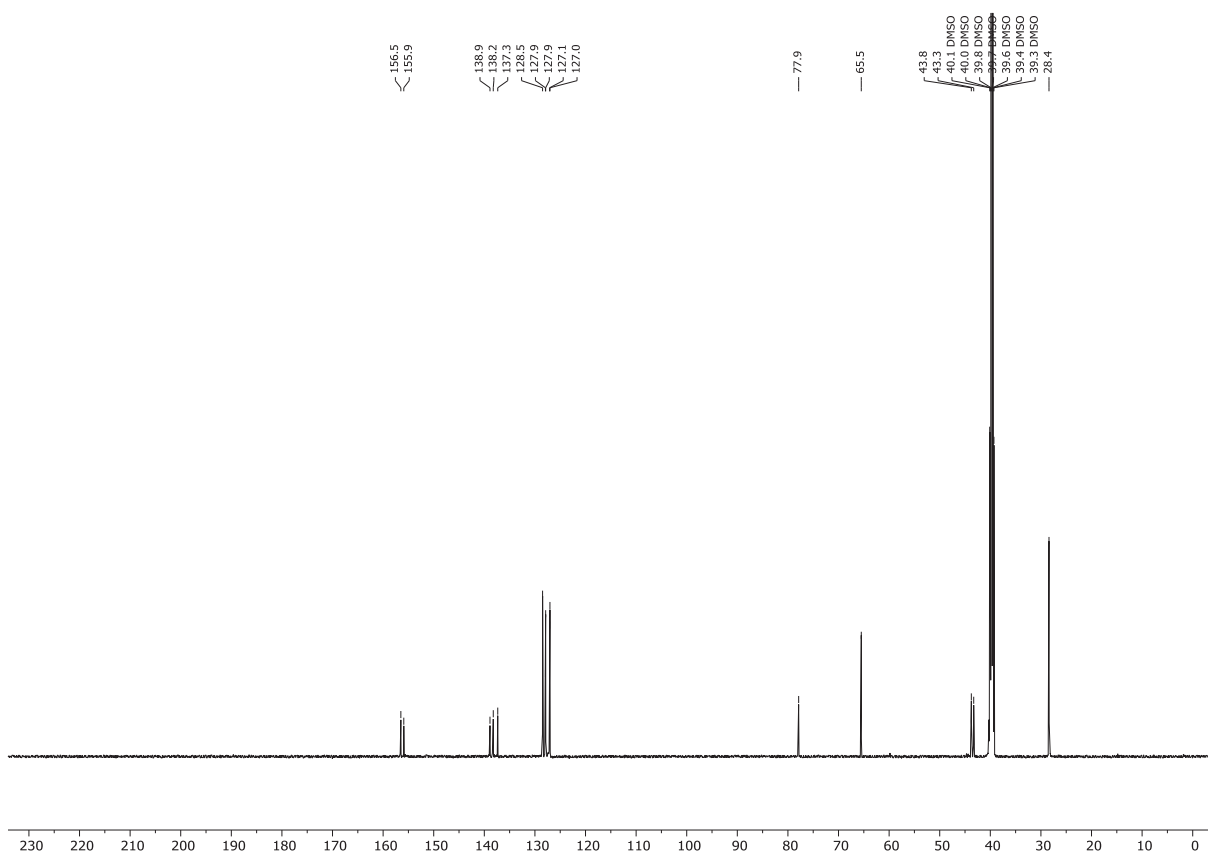
^{19}F NMR spectrum of **15 (565 MHz, $\text{DMSO-}d_6$)**



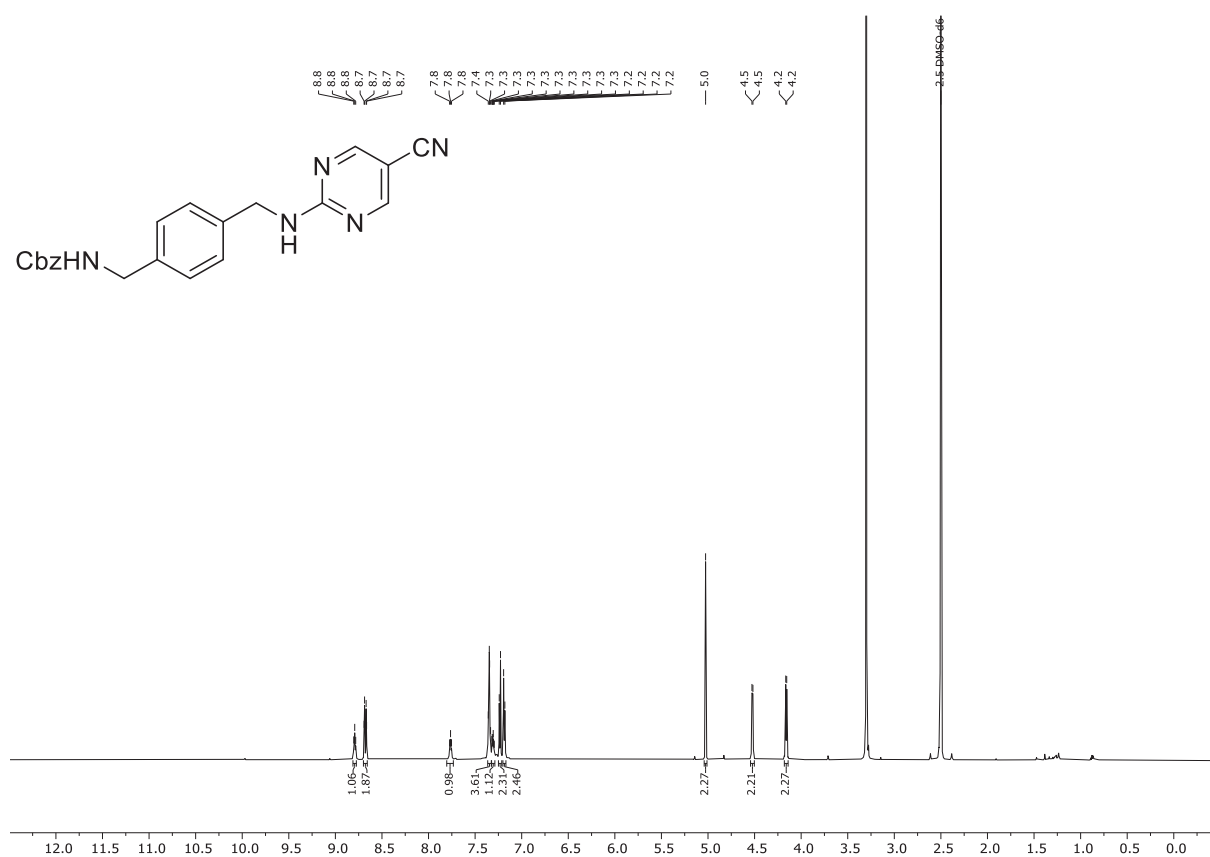
¹H NMR spectrum of **10 (600 MHz, DMSO-*d*₆)**



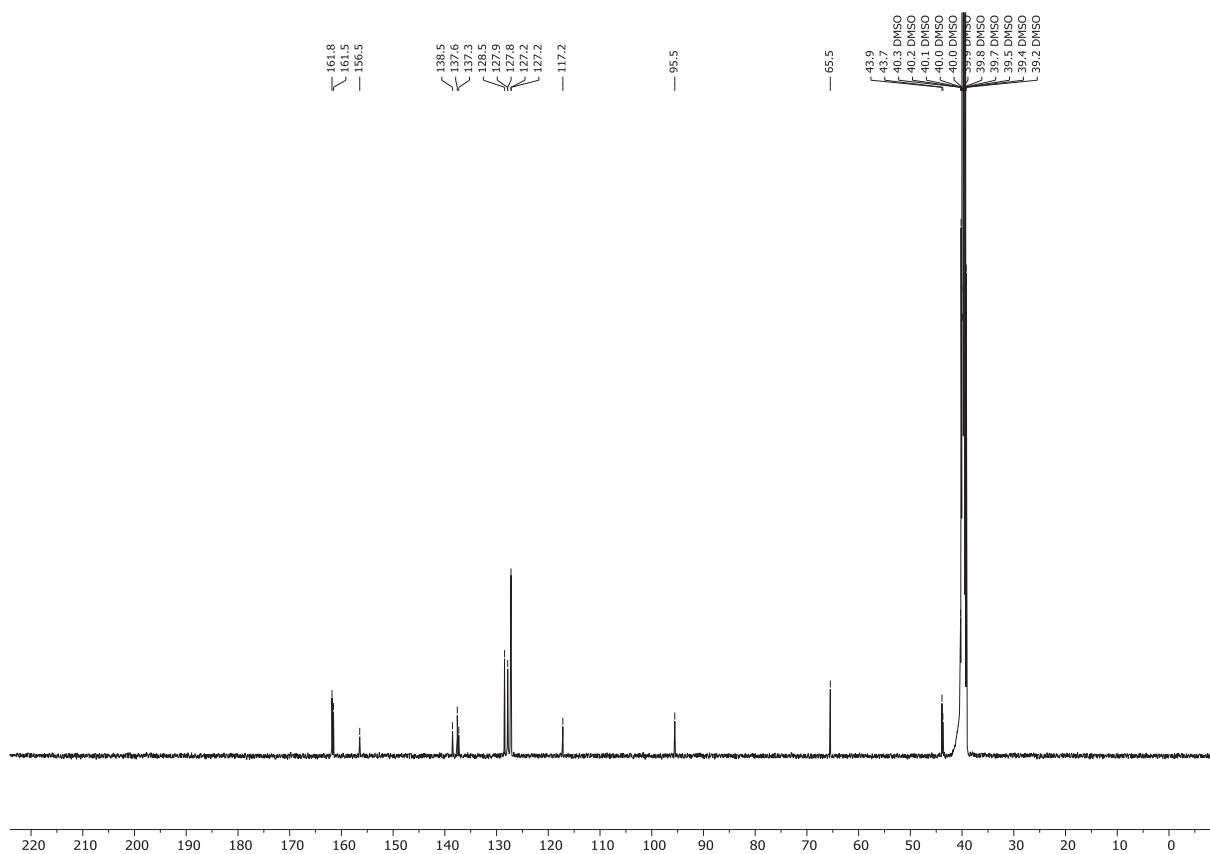
¹³C NMR spectrum of **10 (151 MHz, DMSO-*d*₆)**



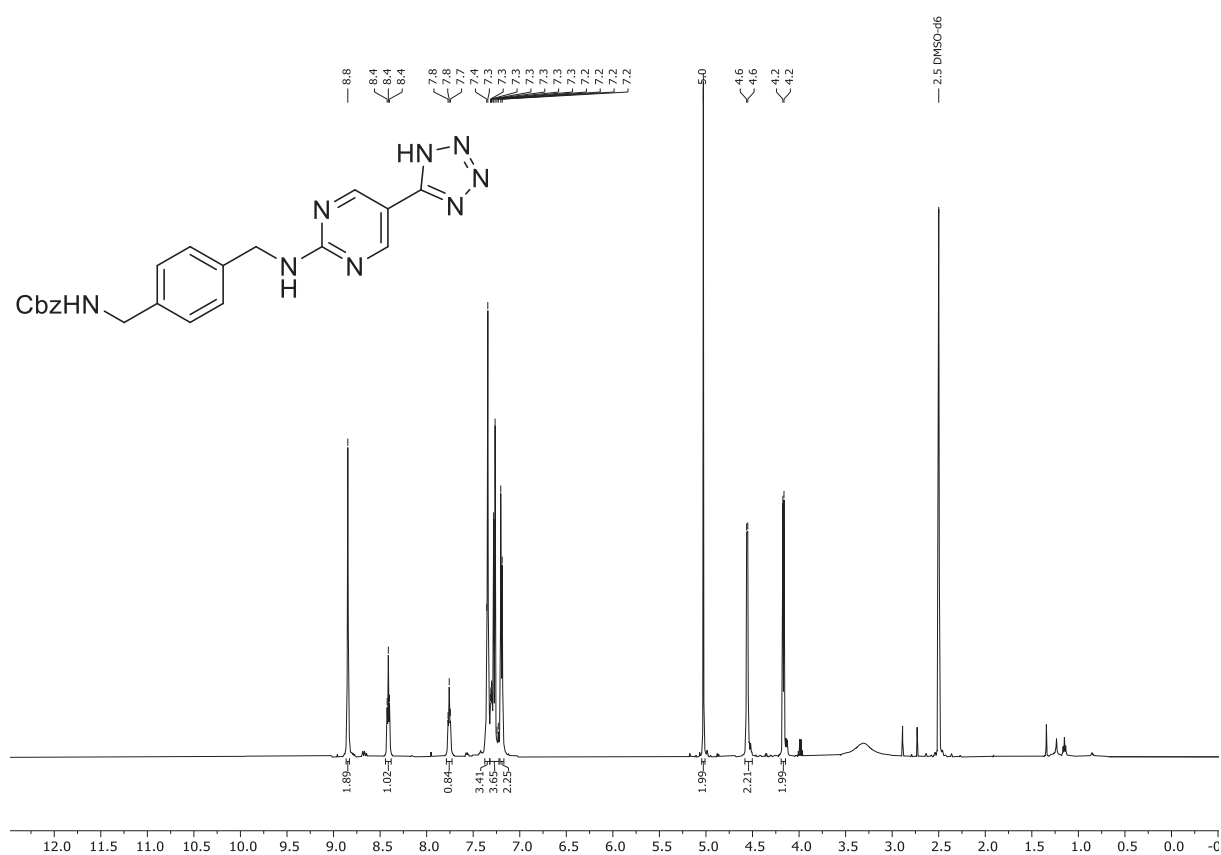
¹H NMR spectrum of **12** (600 MHz, DMSO-*d*₆)



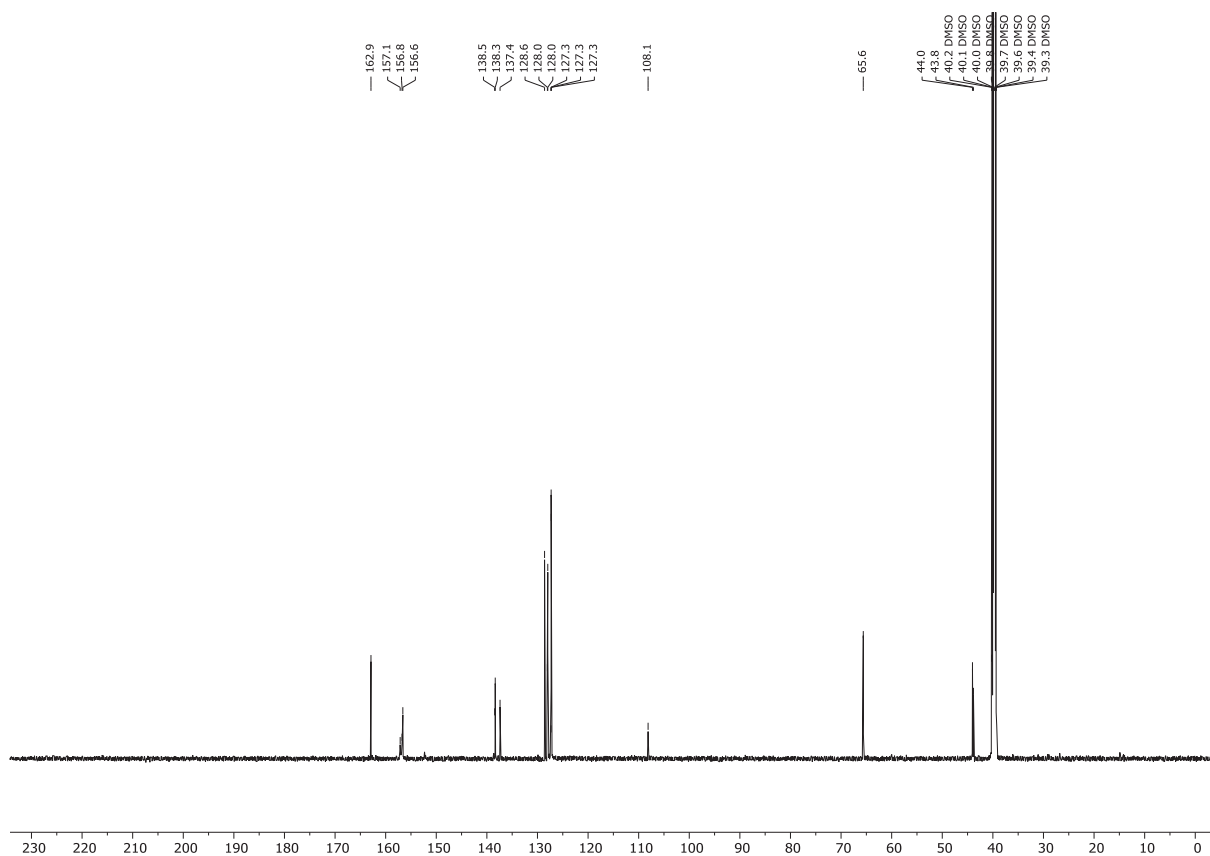
¹³C NMR spectrum of **12** (126 MHz, DMSO-*d*₆)



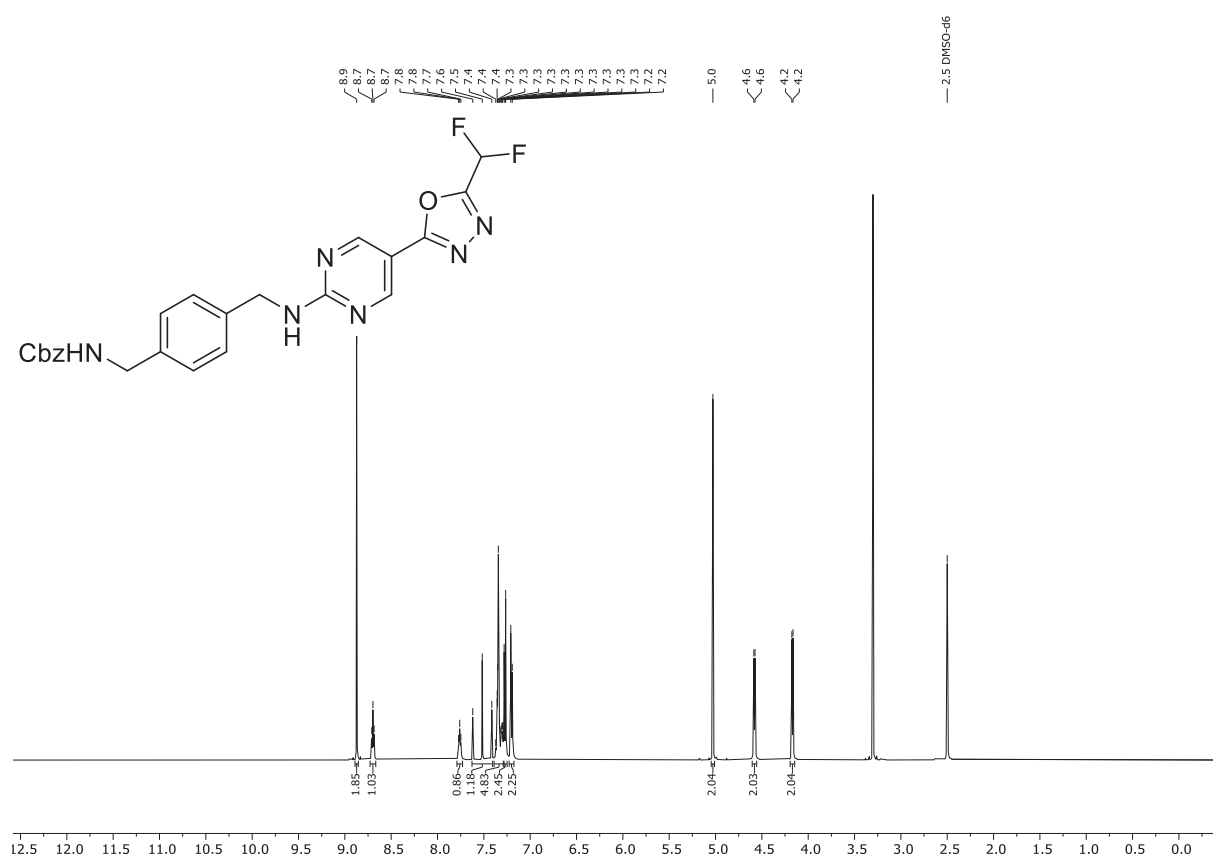
¹H NMR spectrum of **14** (500 MHz, DMSO-*d*₆)



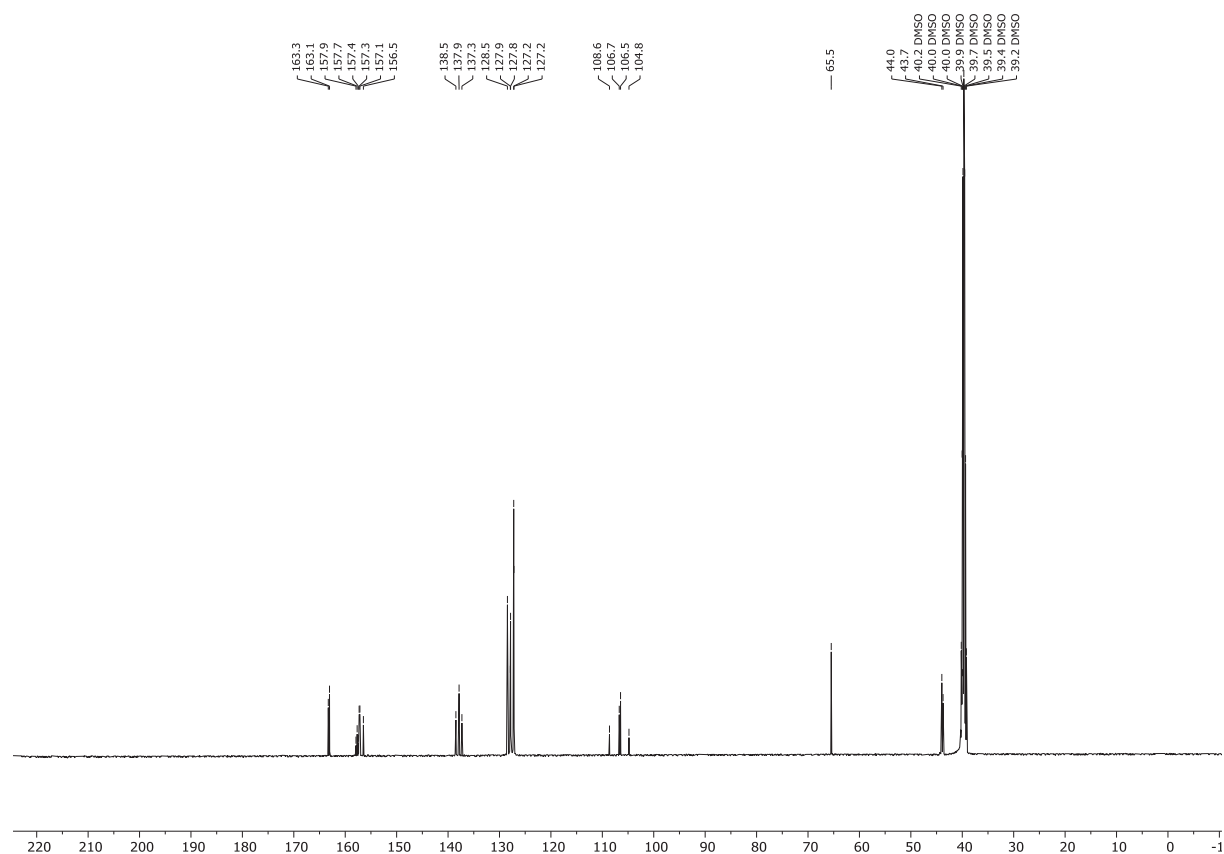
¹³C NMR spectrum of **14** (151 MHz, DMSO-*d*₆)



¹H NMR spectrum of 16 (500 MHz, DMSO-*d*₆)



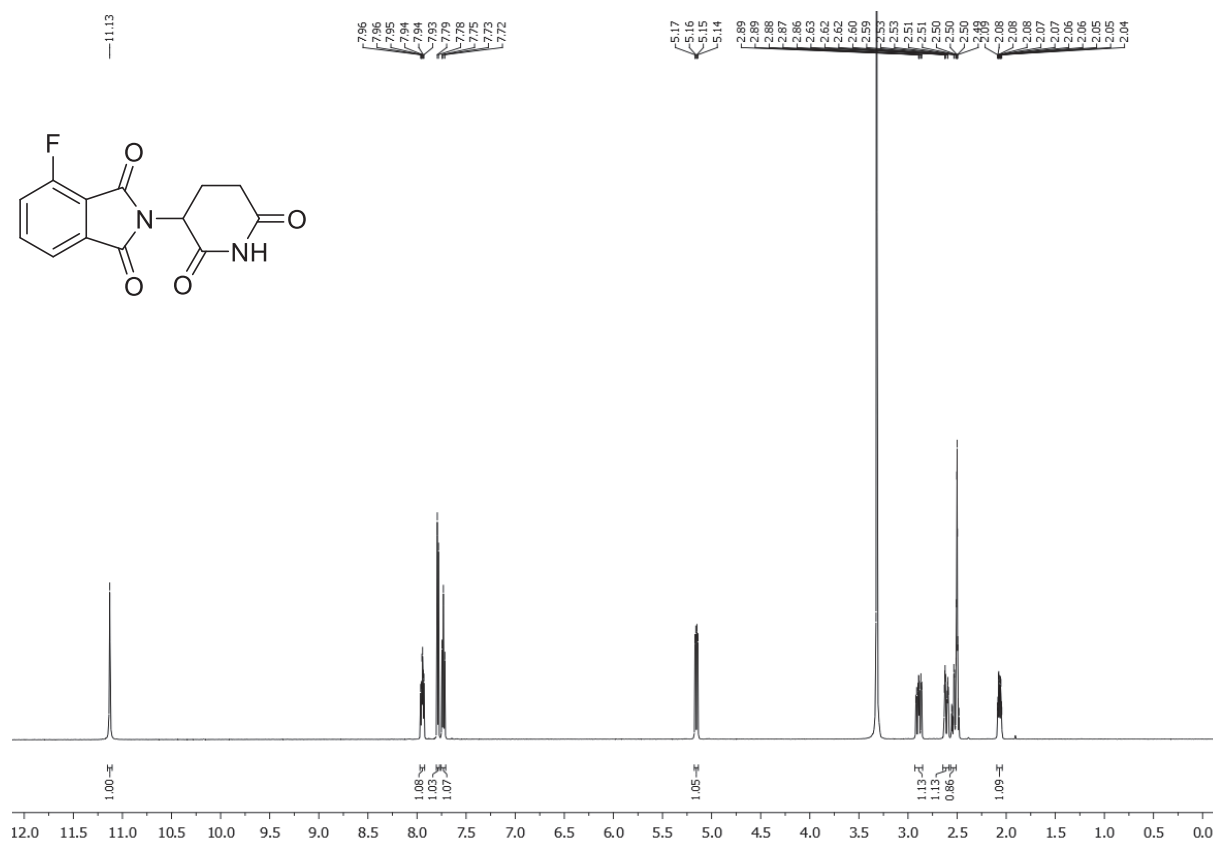
¹³C NMR spectrum of 16 (126 MHz, DMSO-*d*₆)



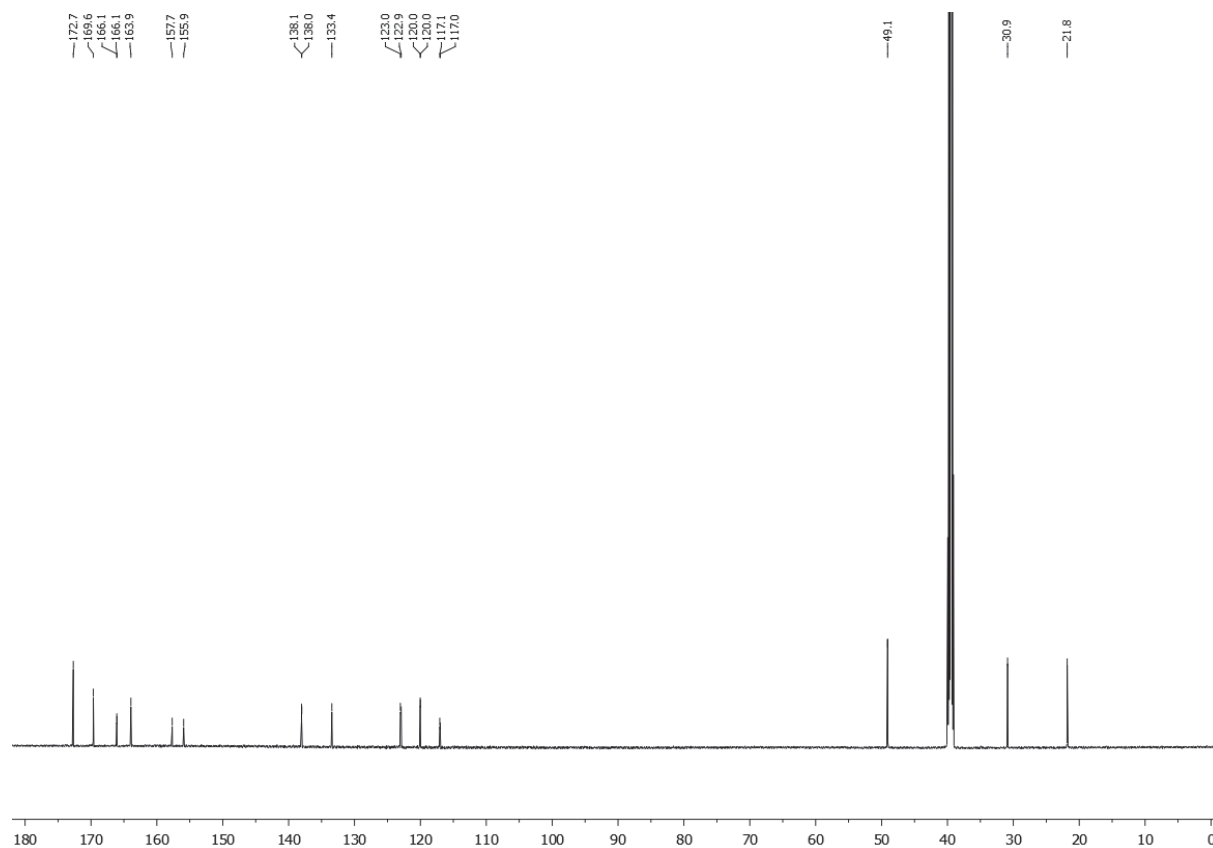
^{19}F NMR spectrum of **16 (565 MHz, $\text{DMSO}-d_6$)**



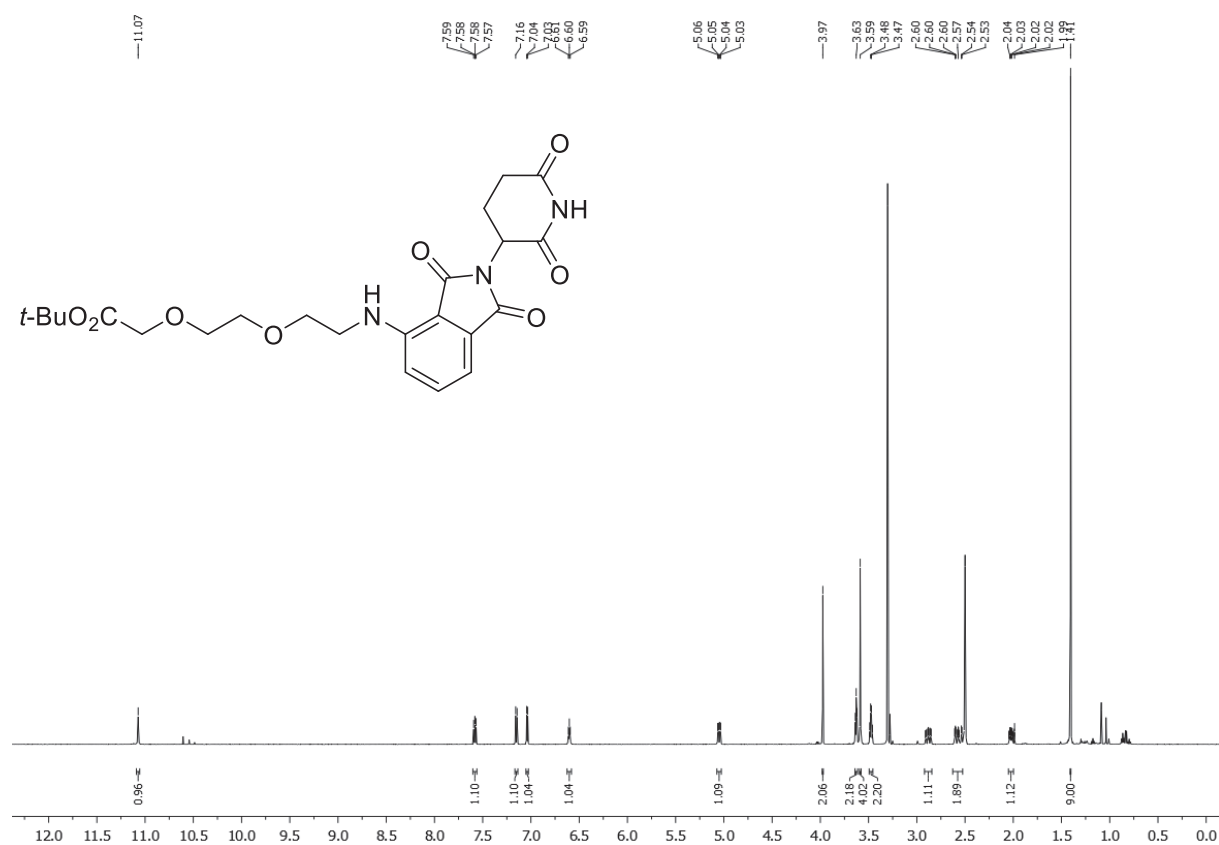
^1H NMR spectrum of **25** (600 MHz, $\text{DMSO-}d_6$)



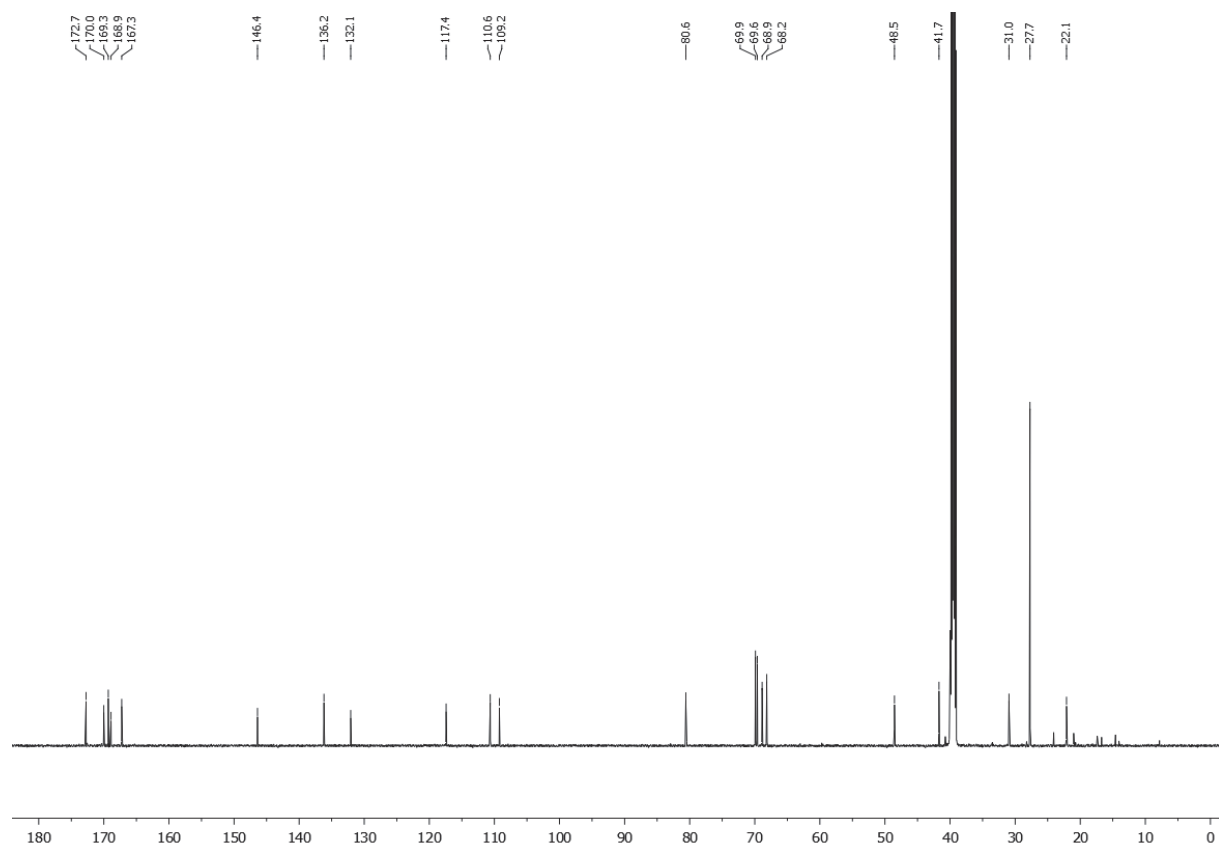
^{13}C NMR spectrum of **25** (151 MHz, $\text{DMSO-}d_6$)



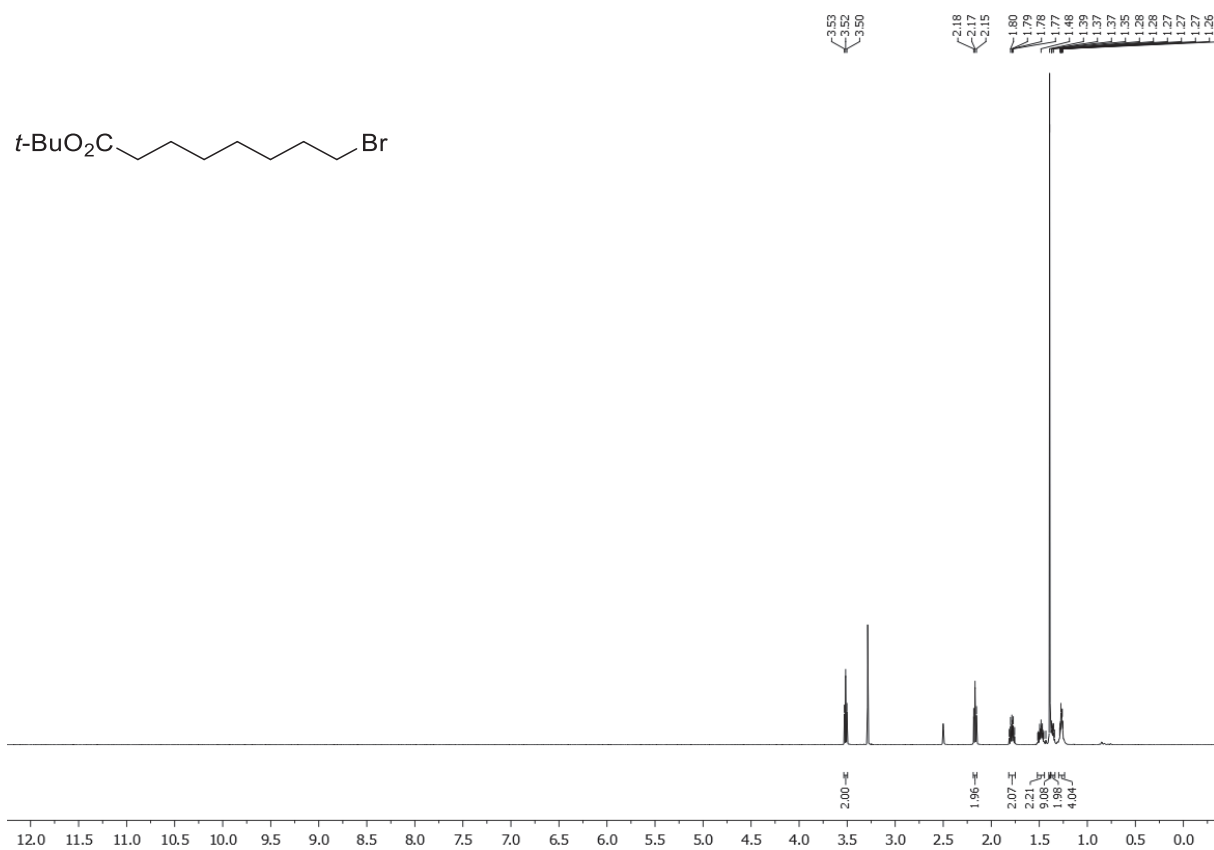
¹H NMR spectrum of **26 (600 MHz, DMSO-*d*₆)**



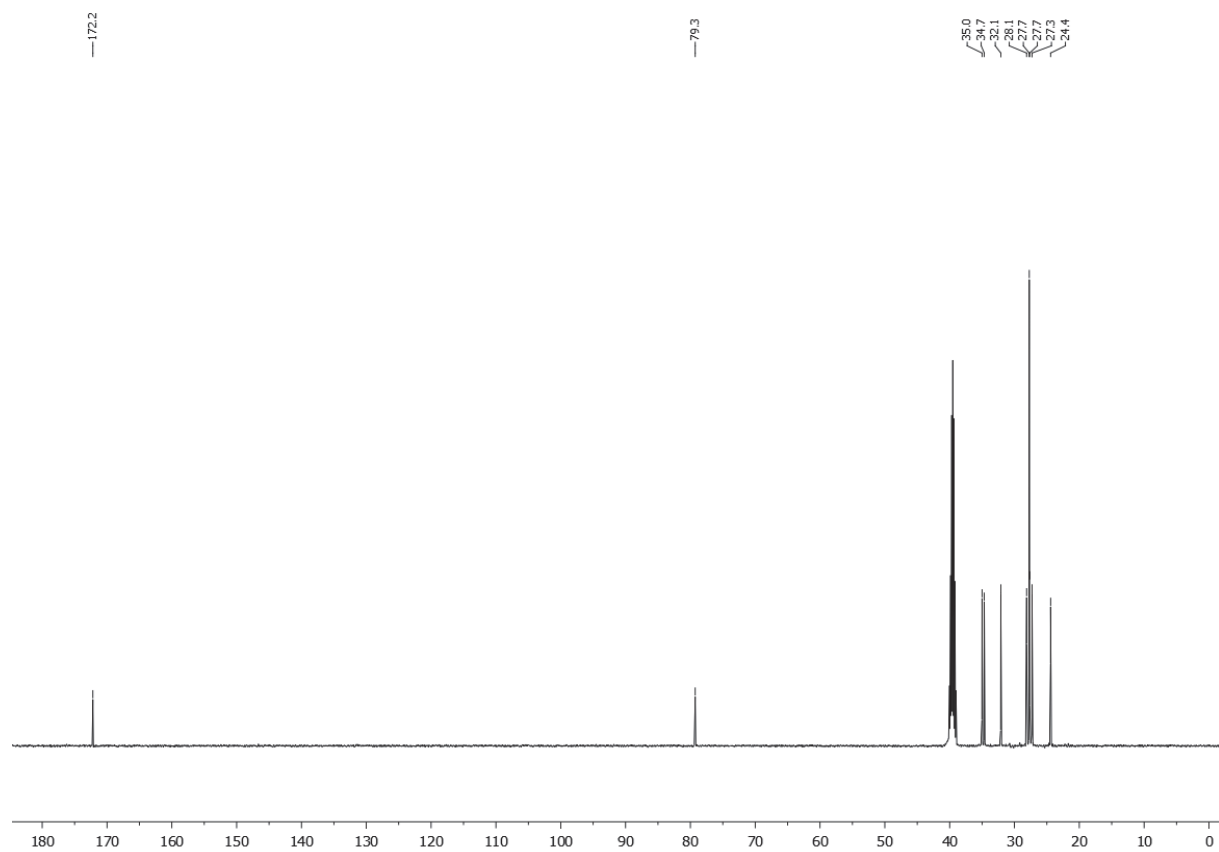
¹³C NMR spectrum of **26 (151 MHz, DMSO-*d*₆)**



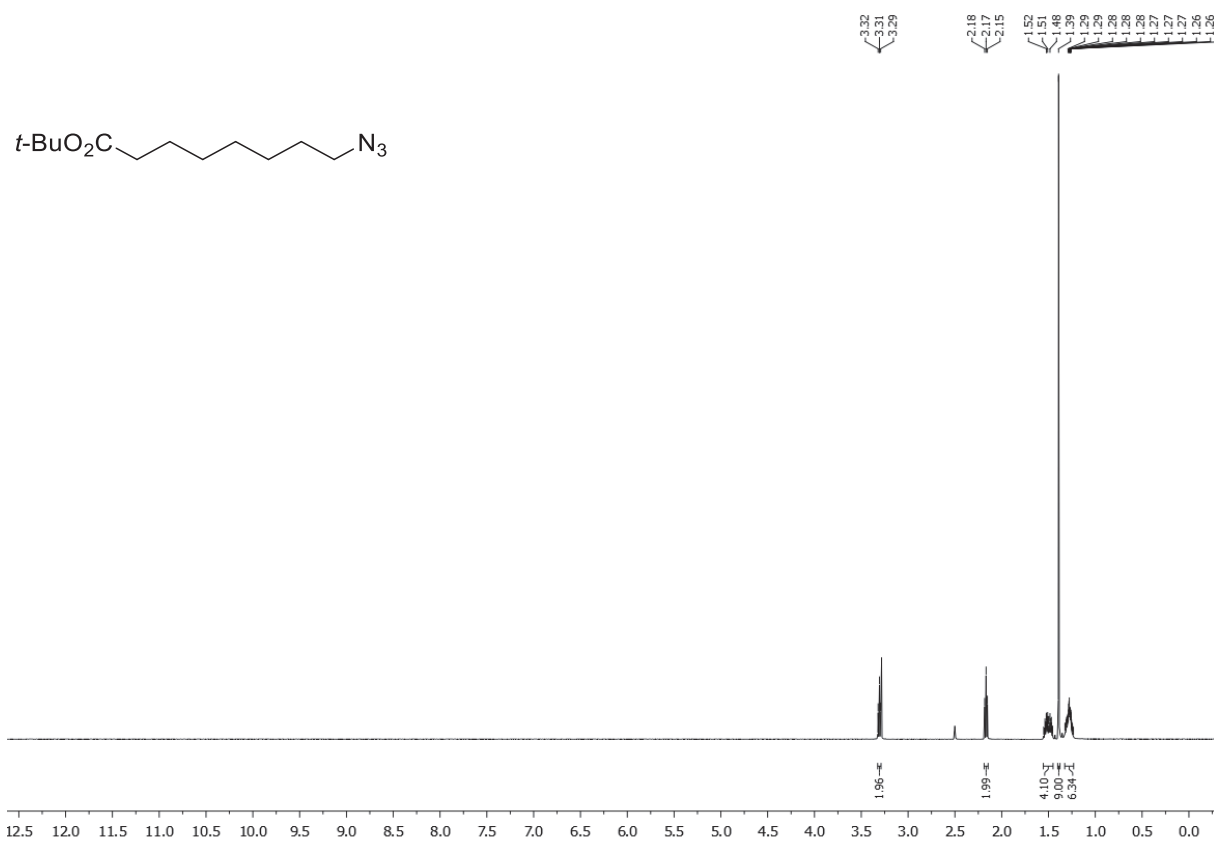
^1H NMR spectrum of **22** (500 MHz, $\text{DMSO}-d_6$)



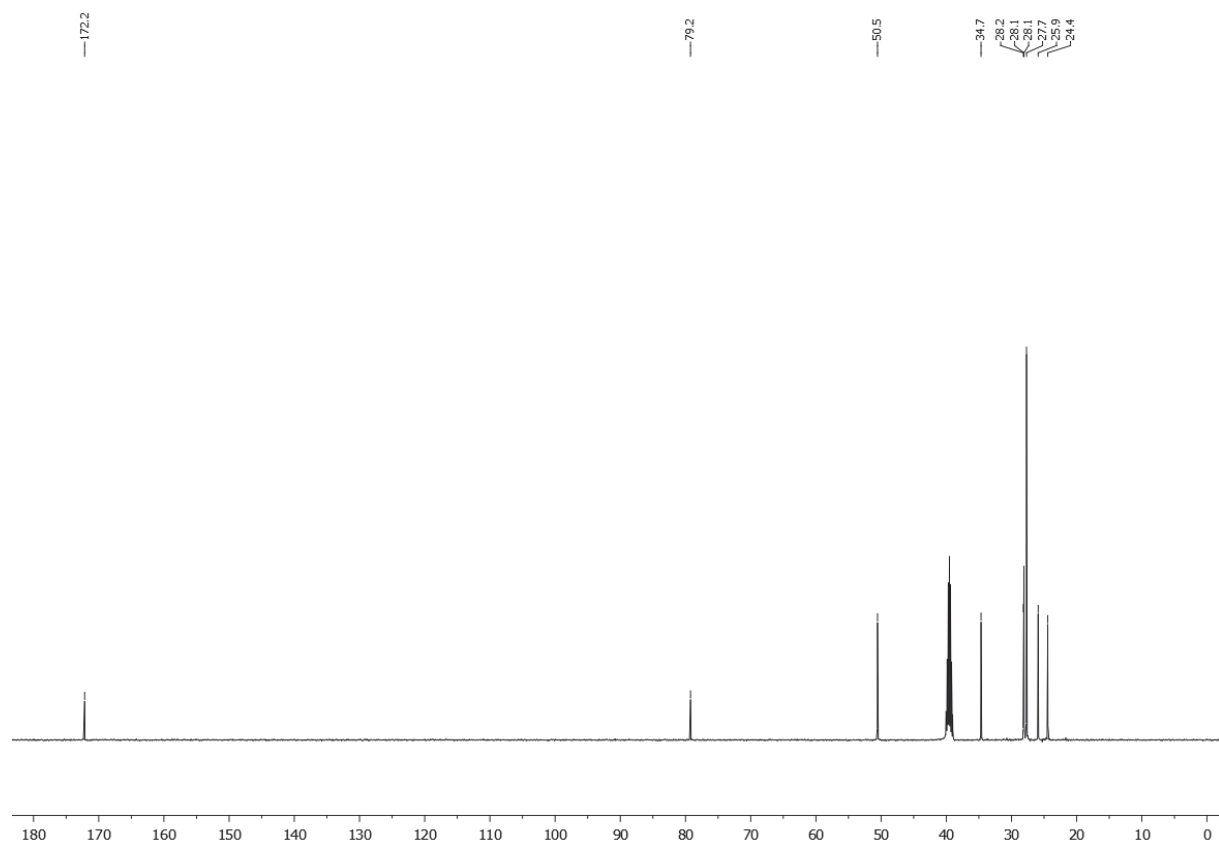
^{13}C NMR spectrum of **22** (126 MHz, $\text{DMSO}-d_6$)



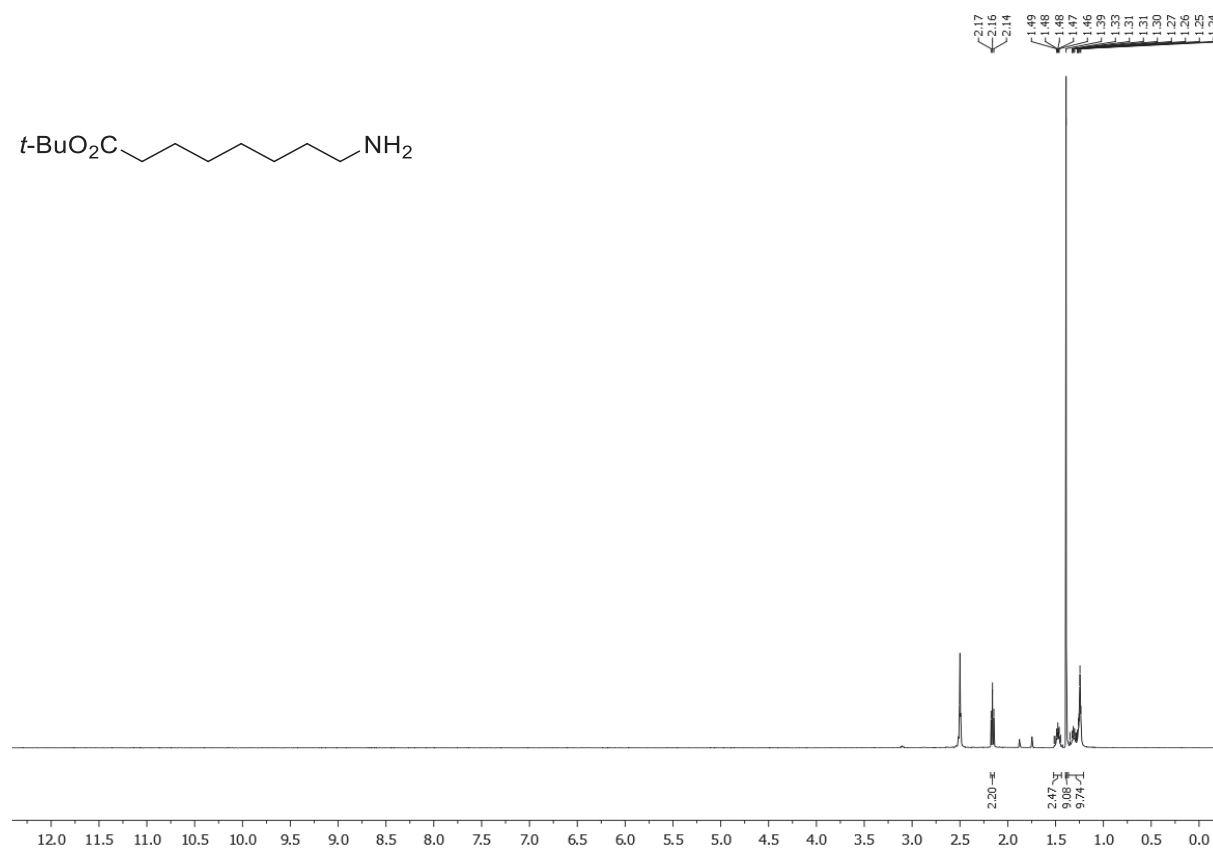
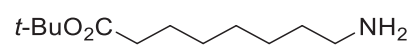
^1H NMR spectrum of **23** (500 MHz, $\text{DMSO}-d_6$)



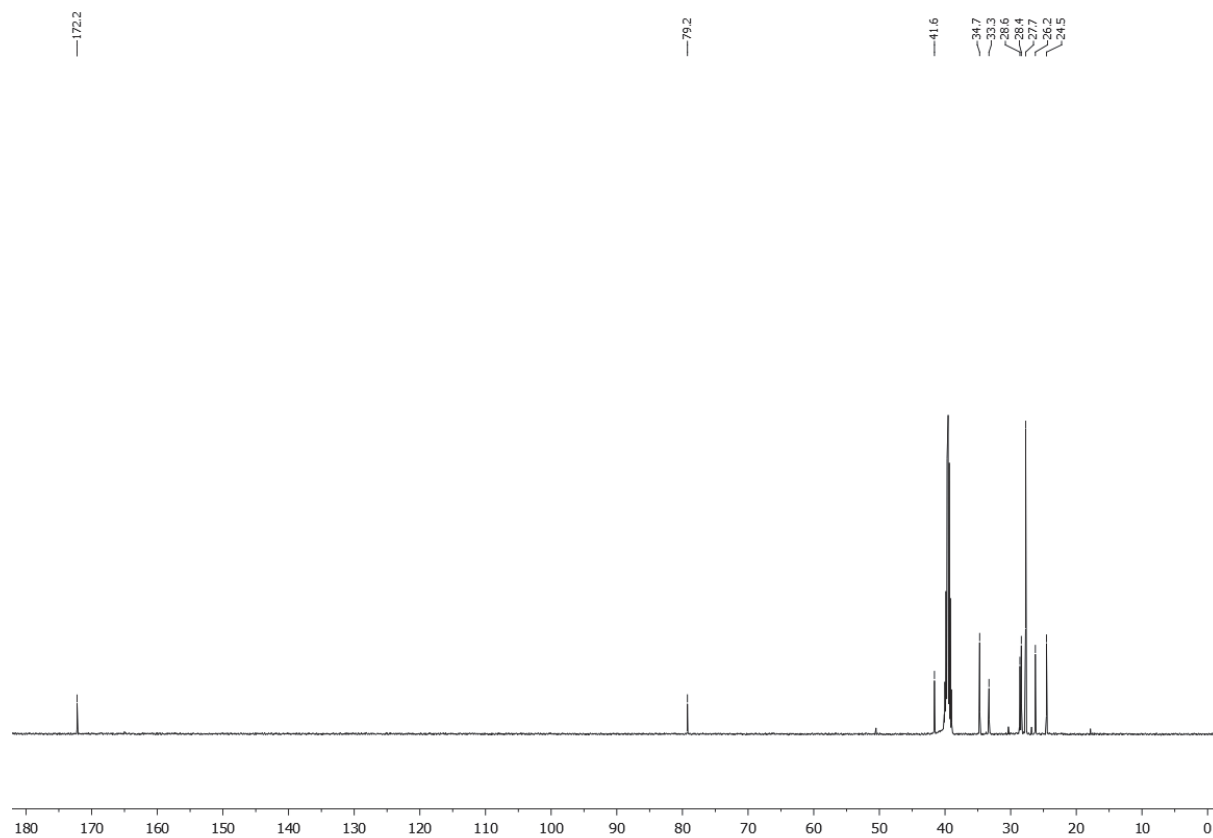
^{13}C NMR spectrum of **23** (126 MHz, $\text{DMSO}-d_6$)



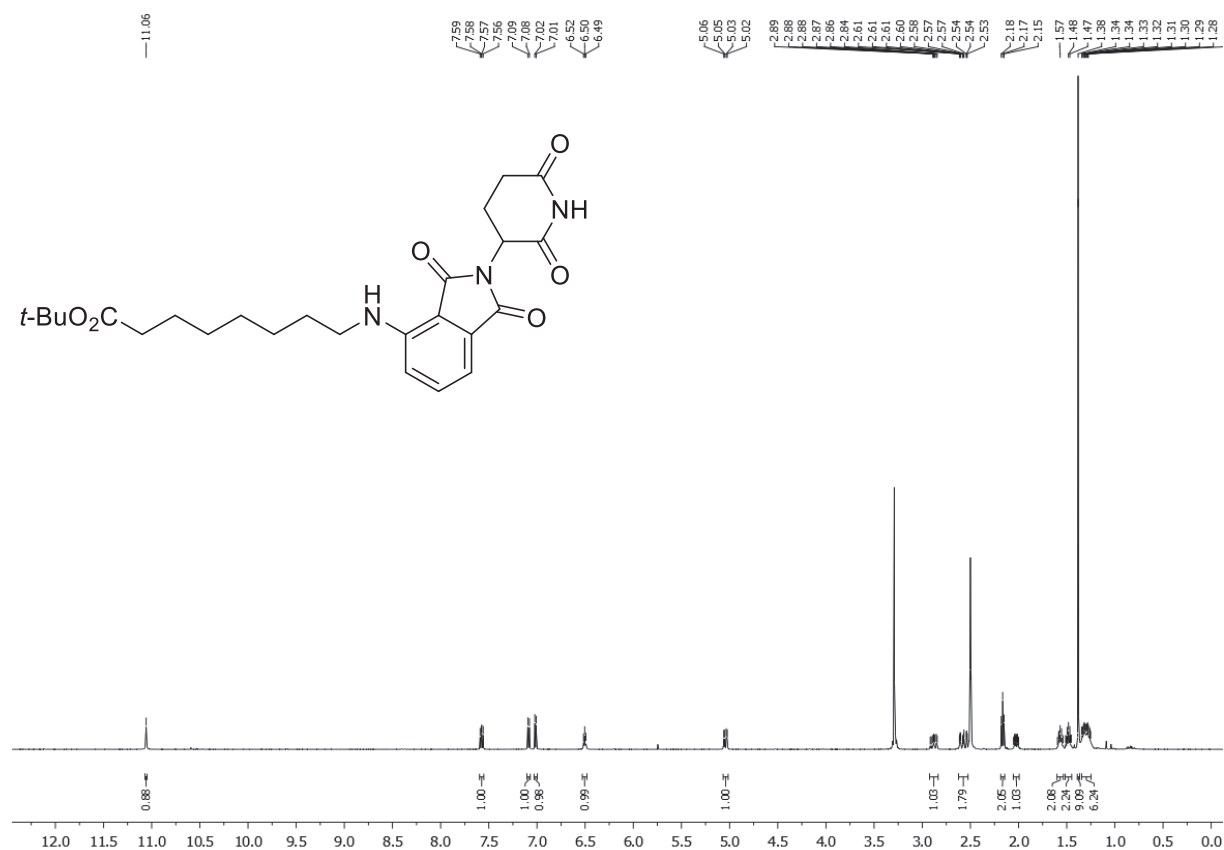
^1H NMR spectrum of **24** (500 MHz, $\text{DMSO}-d_6$)



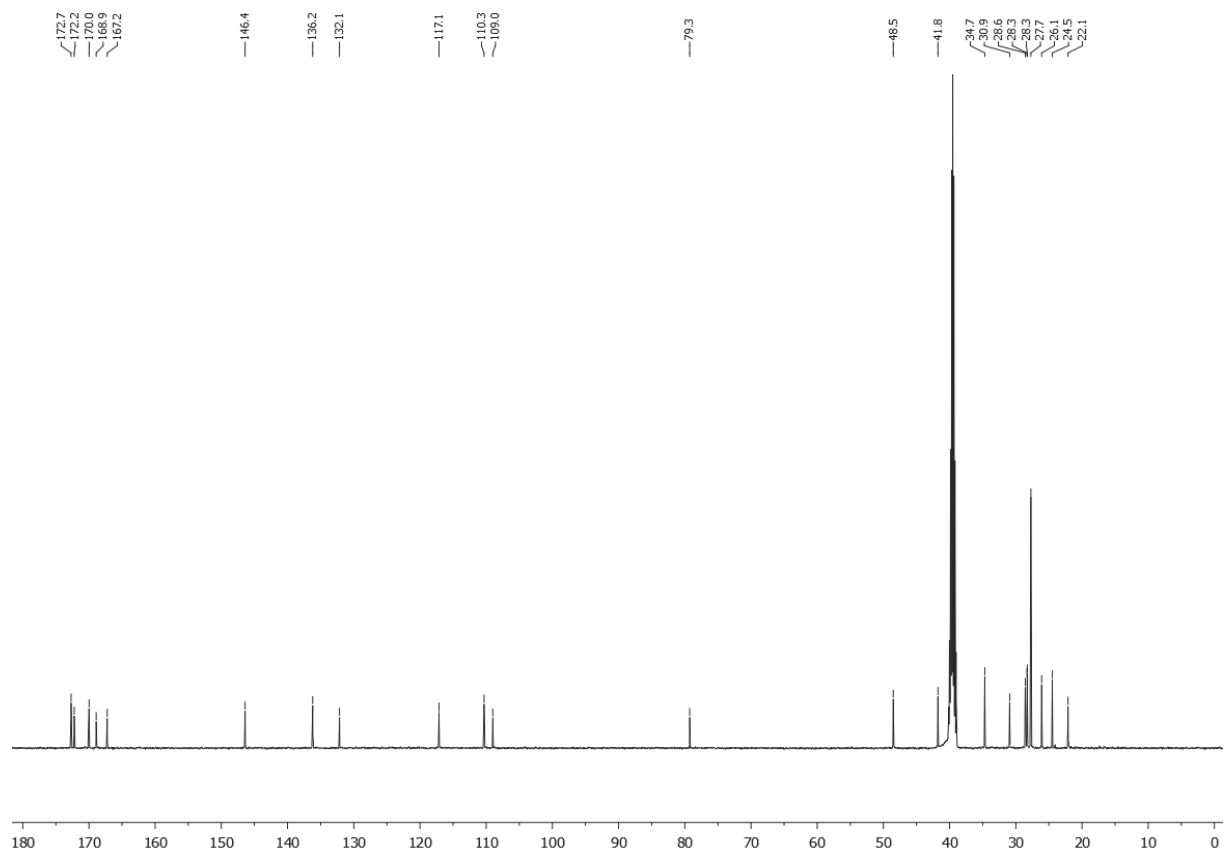
^{13}C NMR spectrum of **24** (126 MHz, $\text{DMSO}-d_6$)



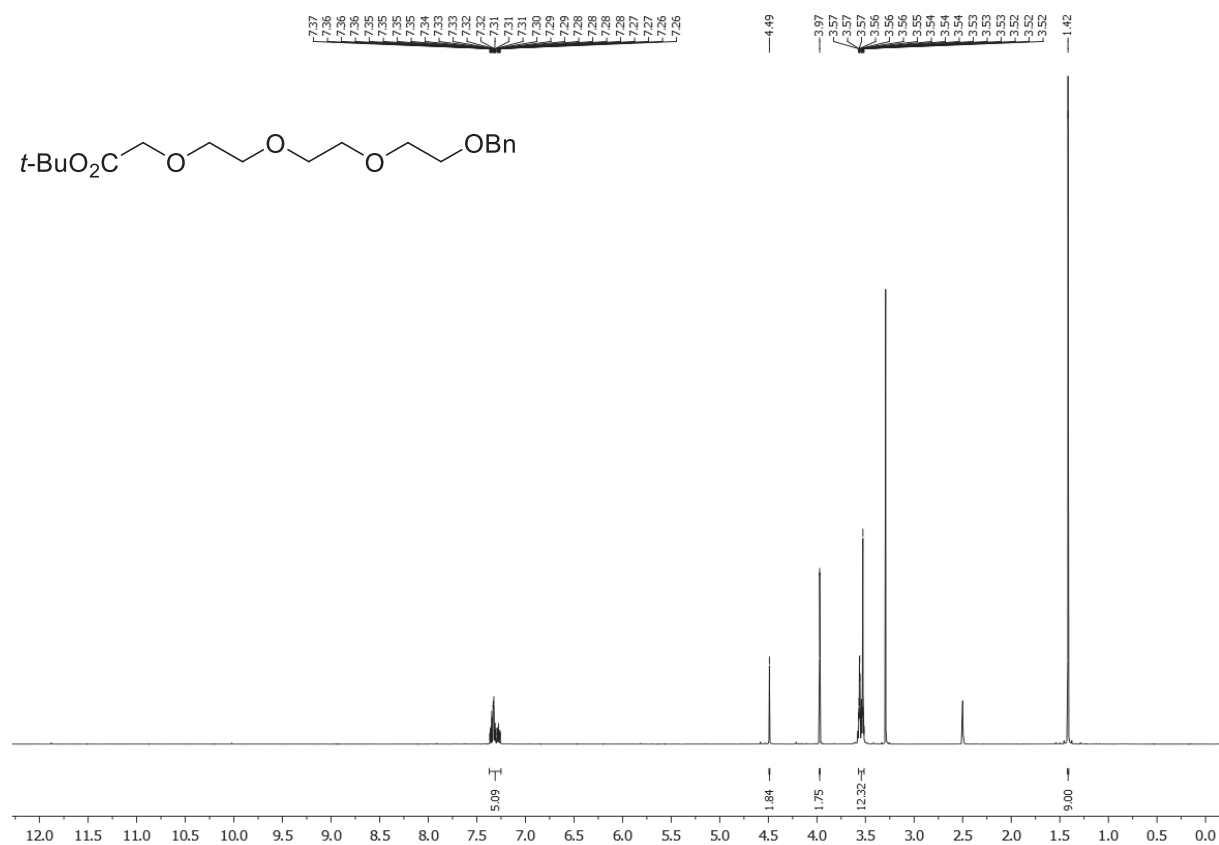
¹H NMR spectrum of **27** (500 MHz, DMSO-*d*₆)



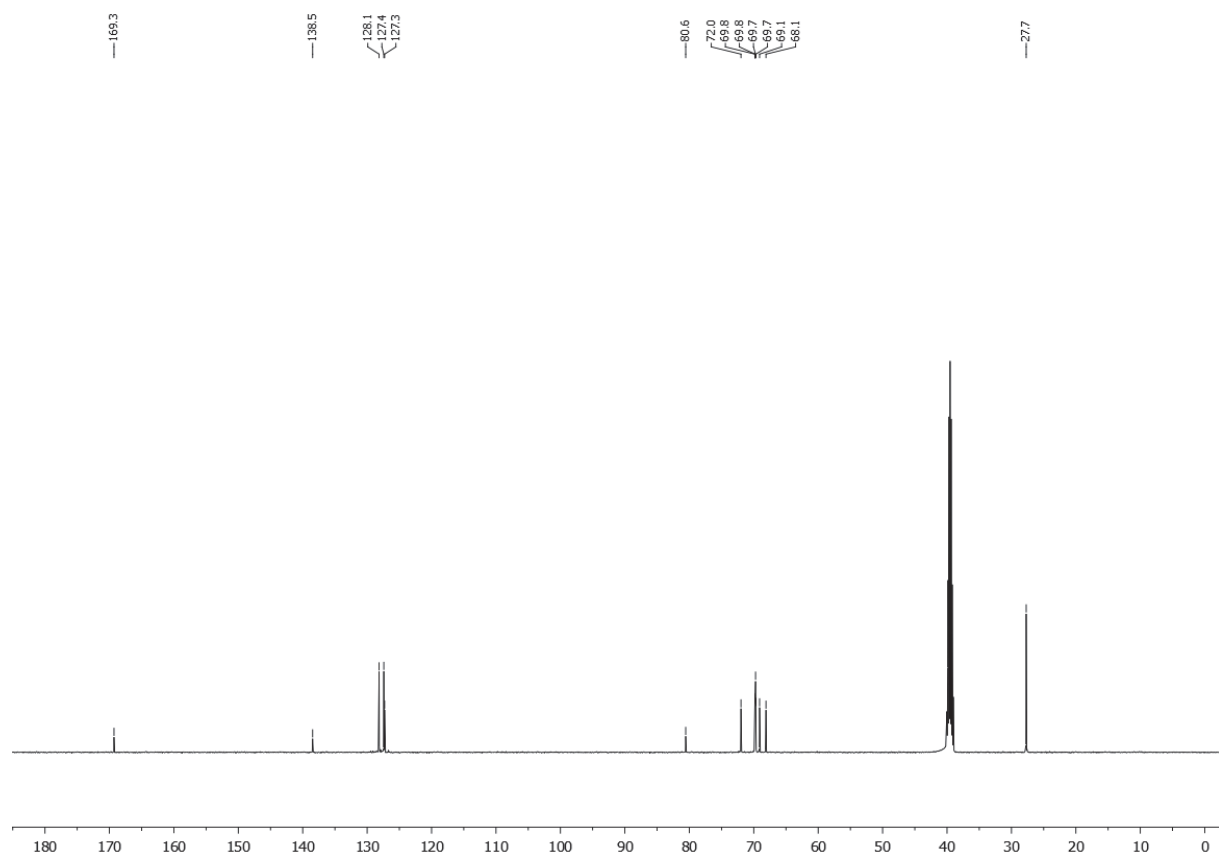
¹³C NMR spectrum of **27** (126 MHz, DMSO-*d*₆)



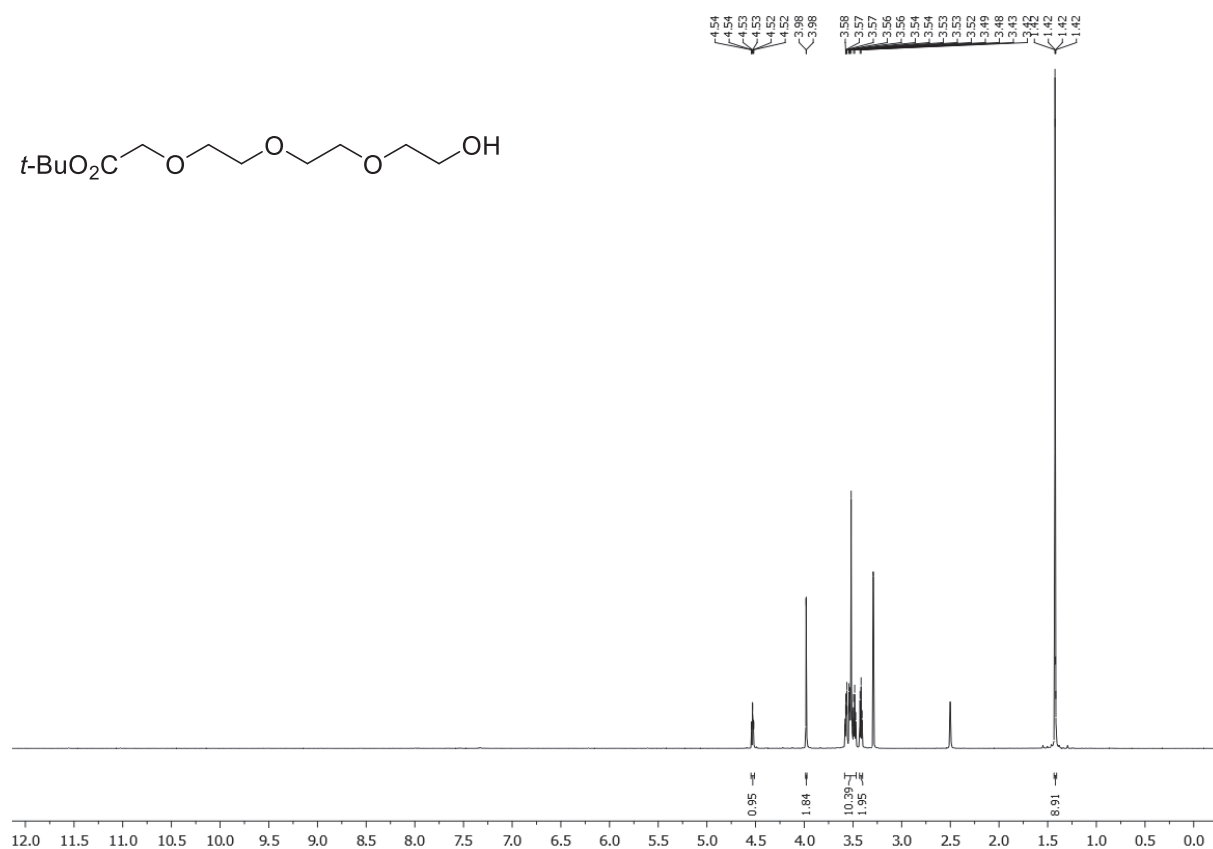
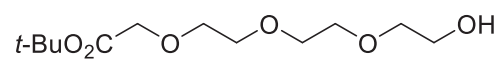
¹H NMR spectrum of **29** (500 MHz, DMSO-*d*₆)



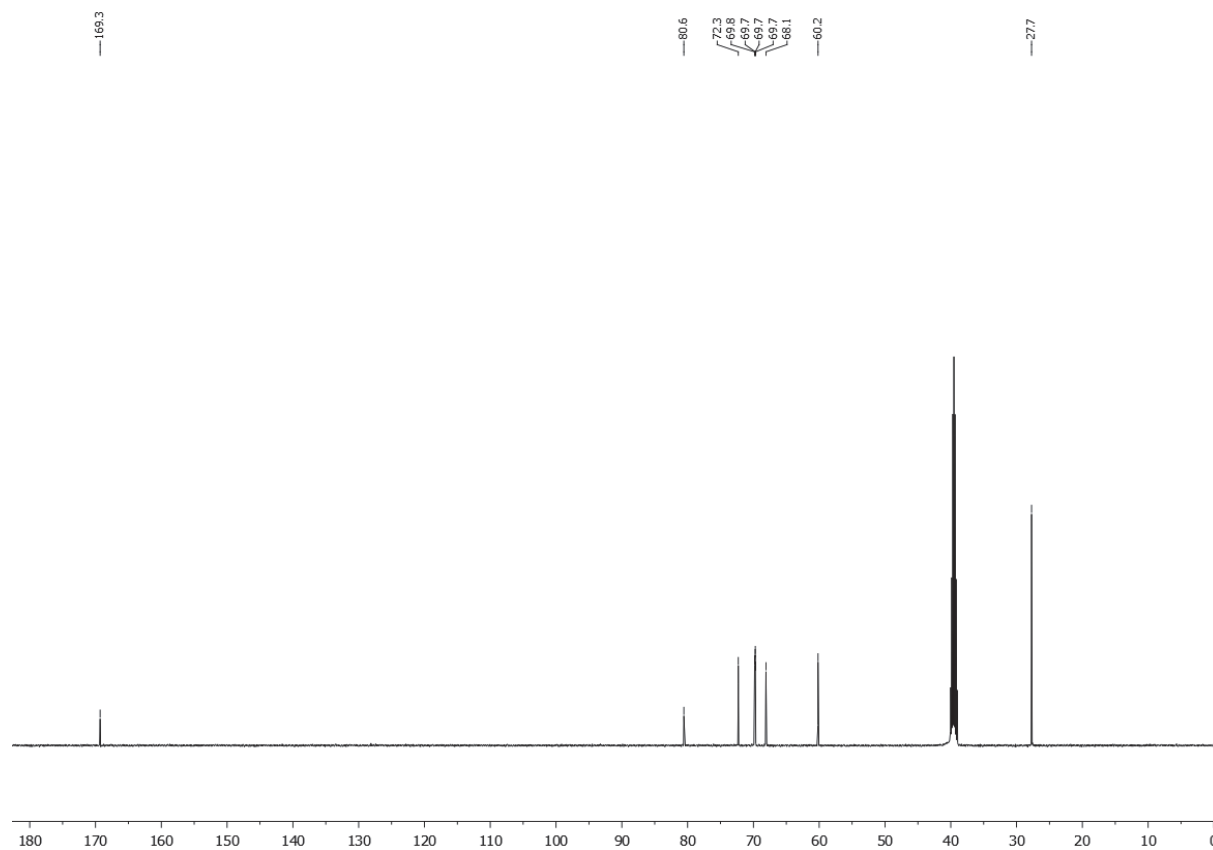
¹³C NMR spectrum of **29** (126 MHz, DMSO-*d*₆)



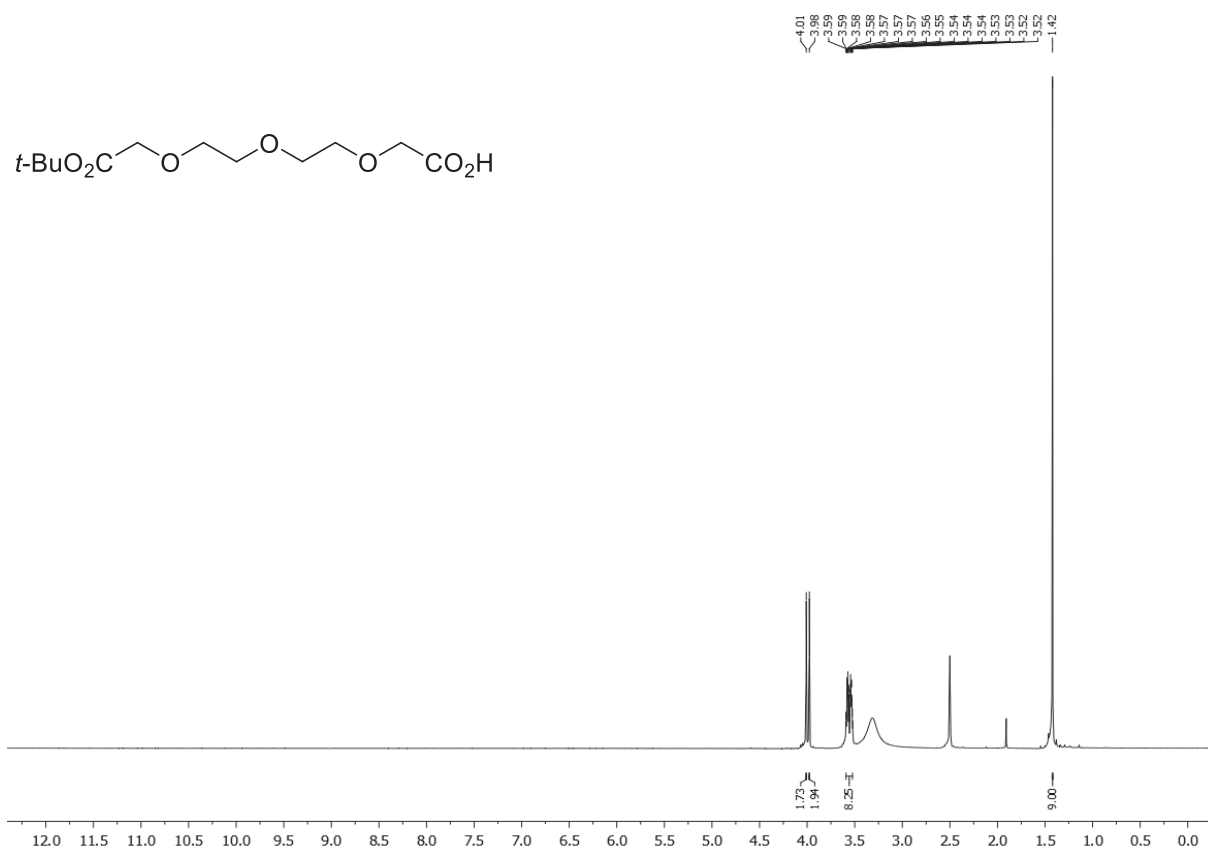
^1H NMR spectrum of **30** (500 MHz, $\text{DMSO-}d_6$)



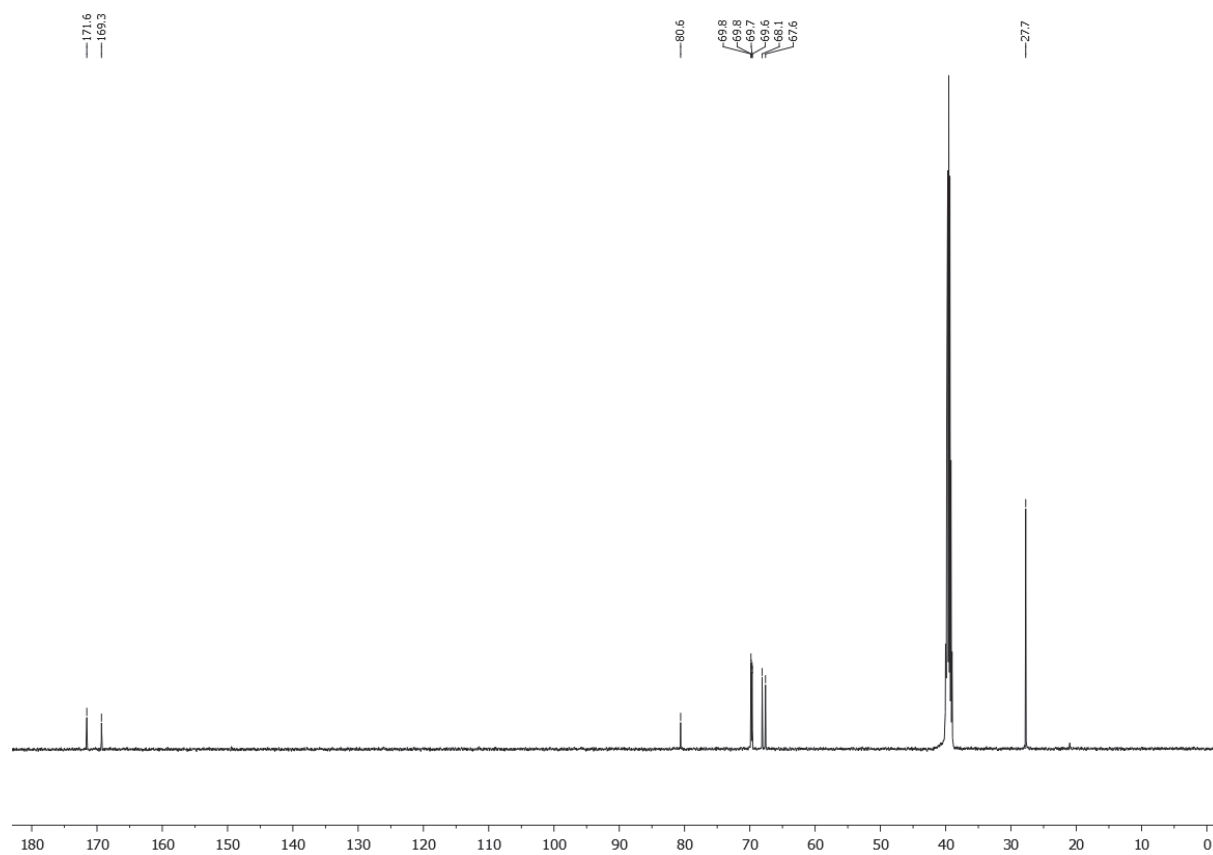
^{13}C NMR spectrum of **30** (126 MHz, $\text{DMSO-}d_6$)



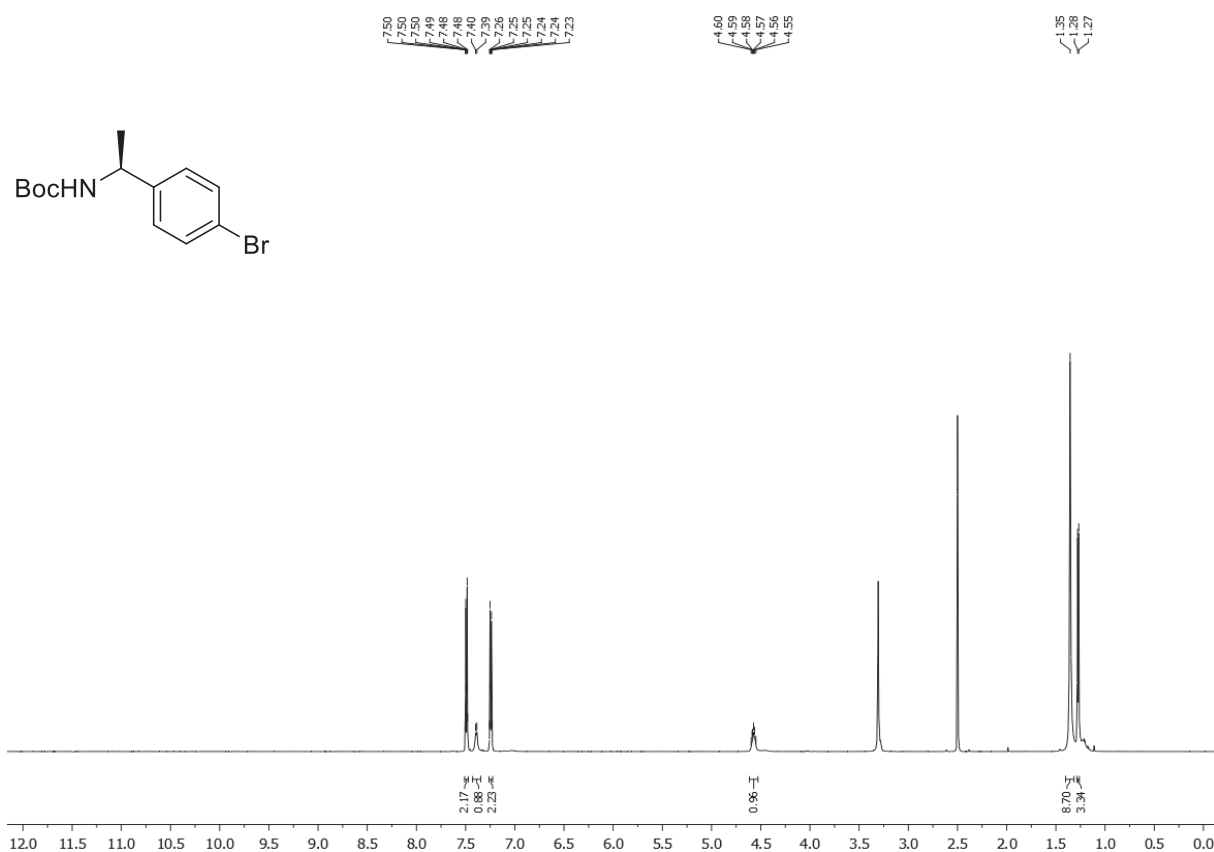
¹H NMR spectrum of **31** (500 MHz, DMSO-*d*₆)



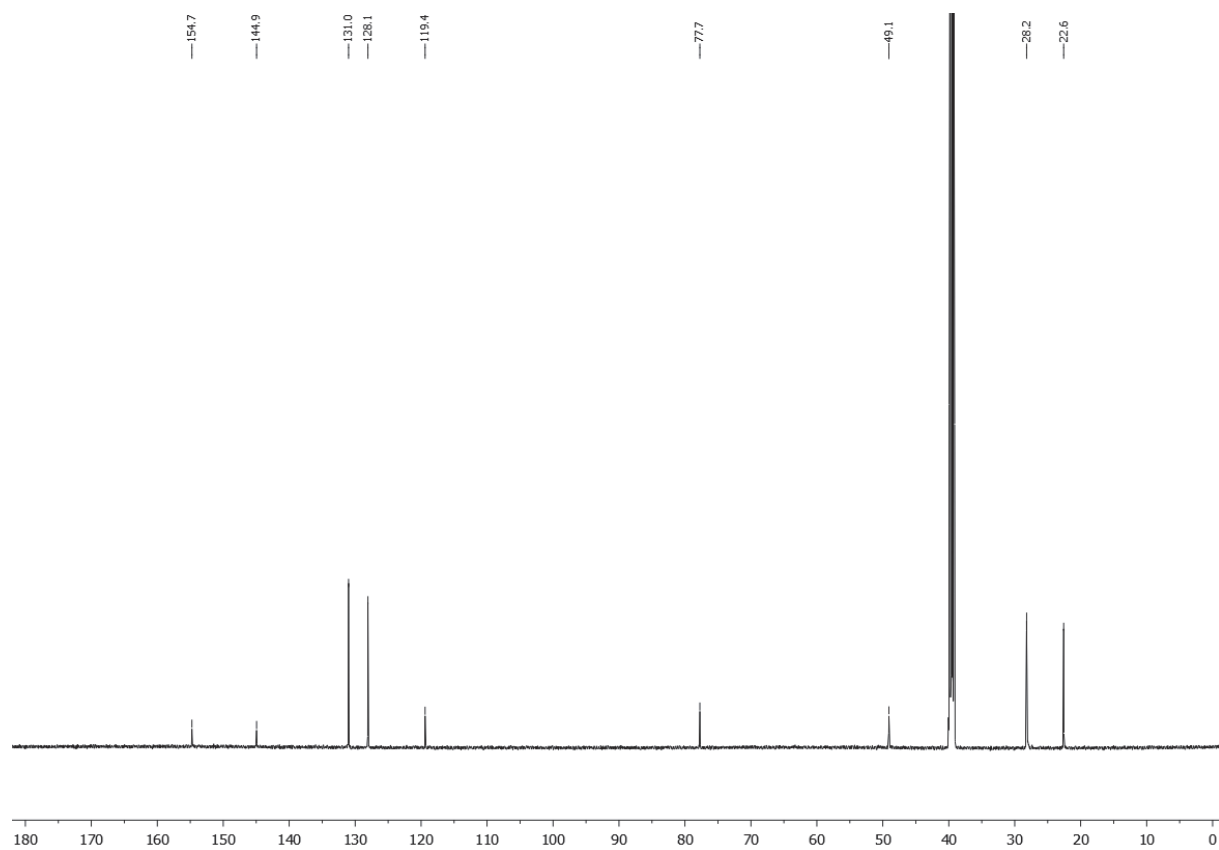
¹³C NMR spectrum of **31** (126 MHz, DMSO-*d*₆)



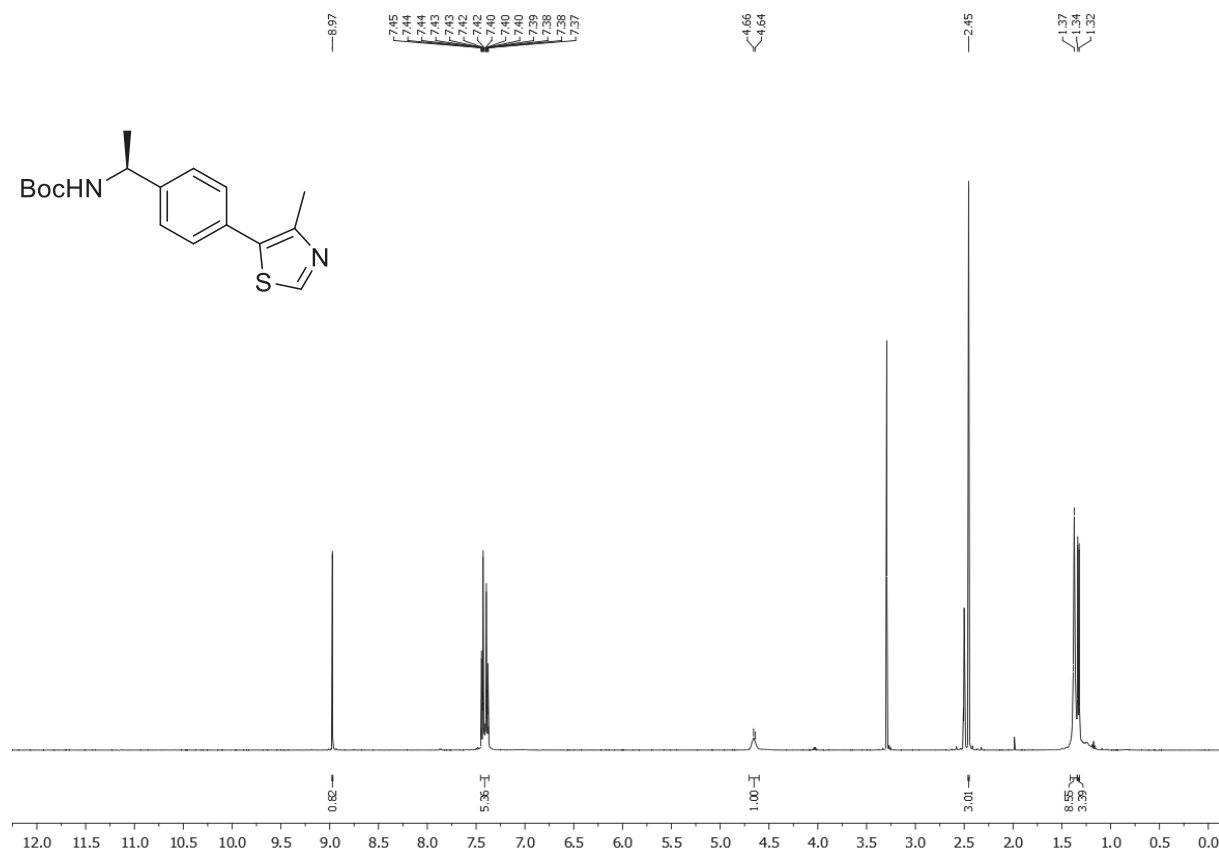
^1H NMR spectrum of **37** (600 MHz, $\text{DMSO-}d_6$)



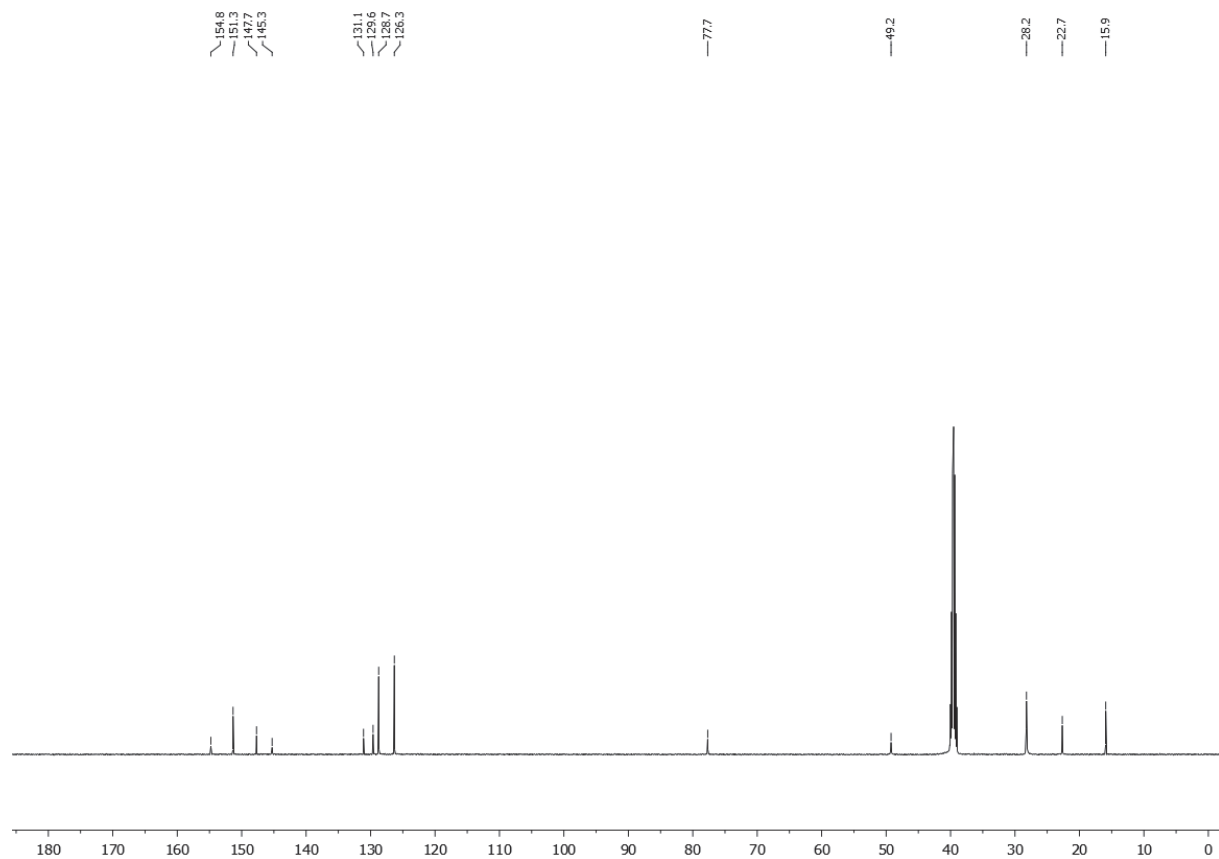
^{13}C NMR spectrum of **37** (151 MHz, $\text{DMSO-}d_6$)



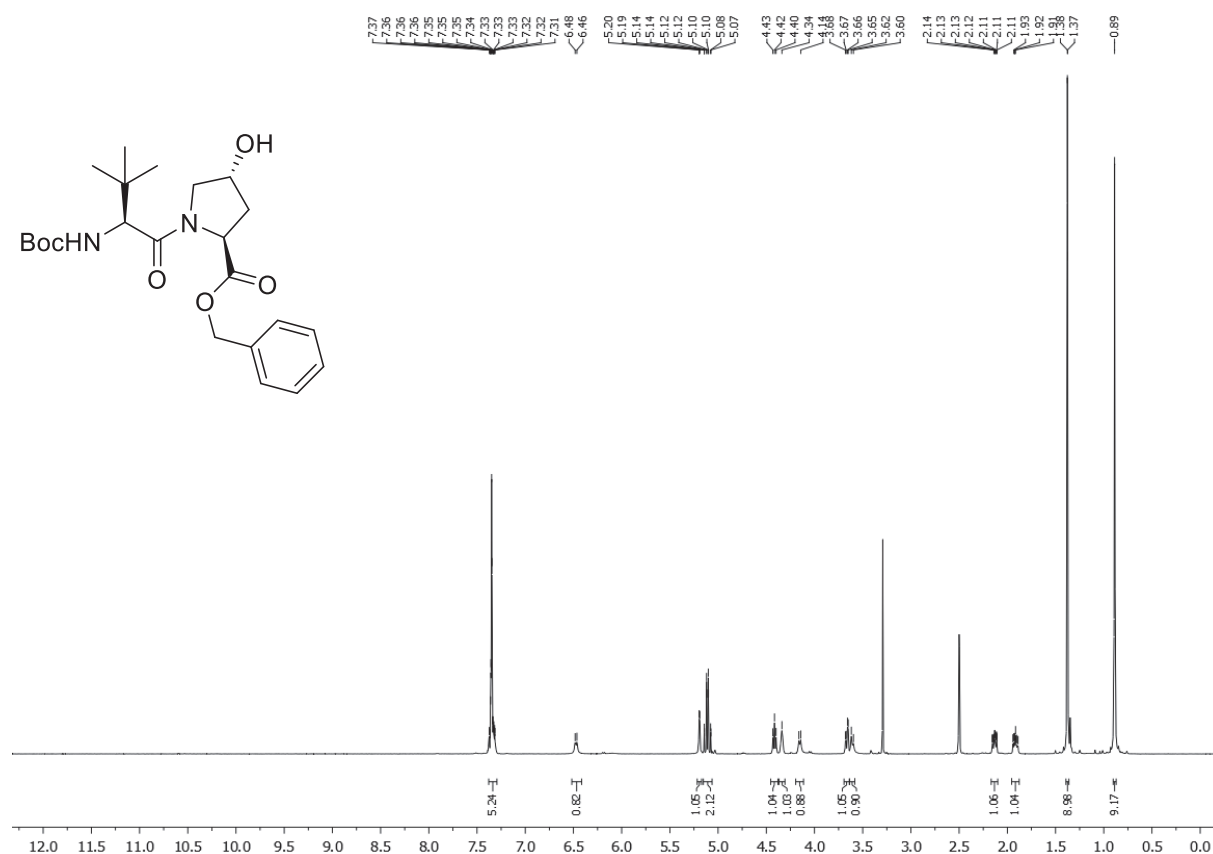
¹H NMR spectrum of **38 (500 MHz, DMSO-*d*₆)**



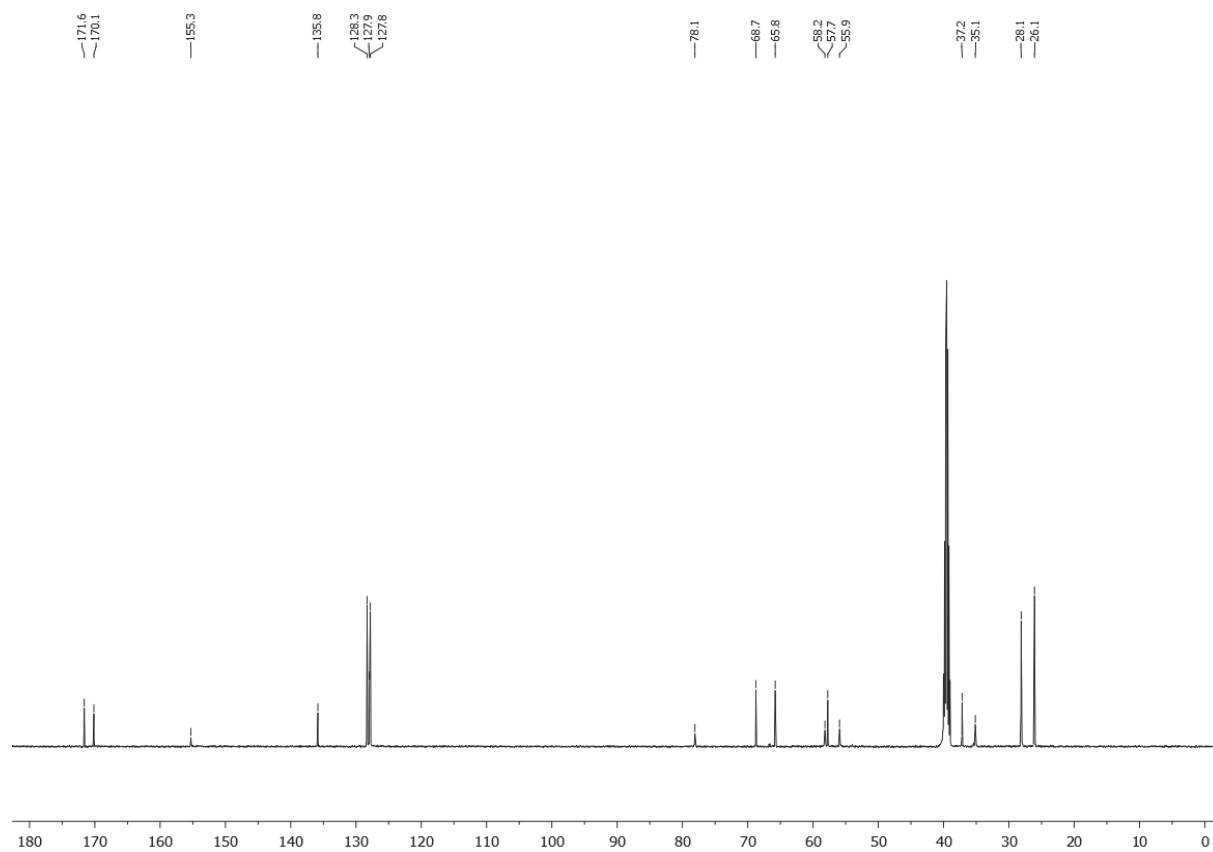
¹³C NMR spectrum of **38 (126 MHz, DMSO-*d*₆)**



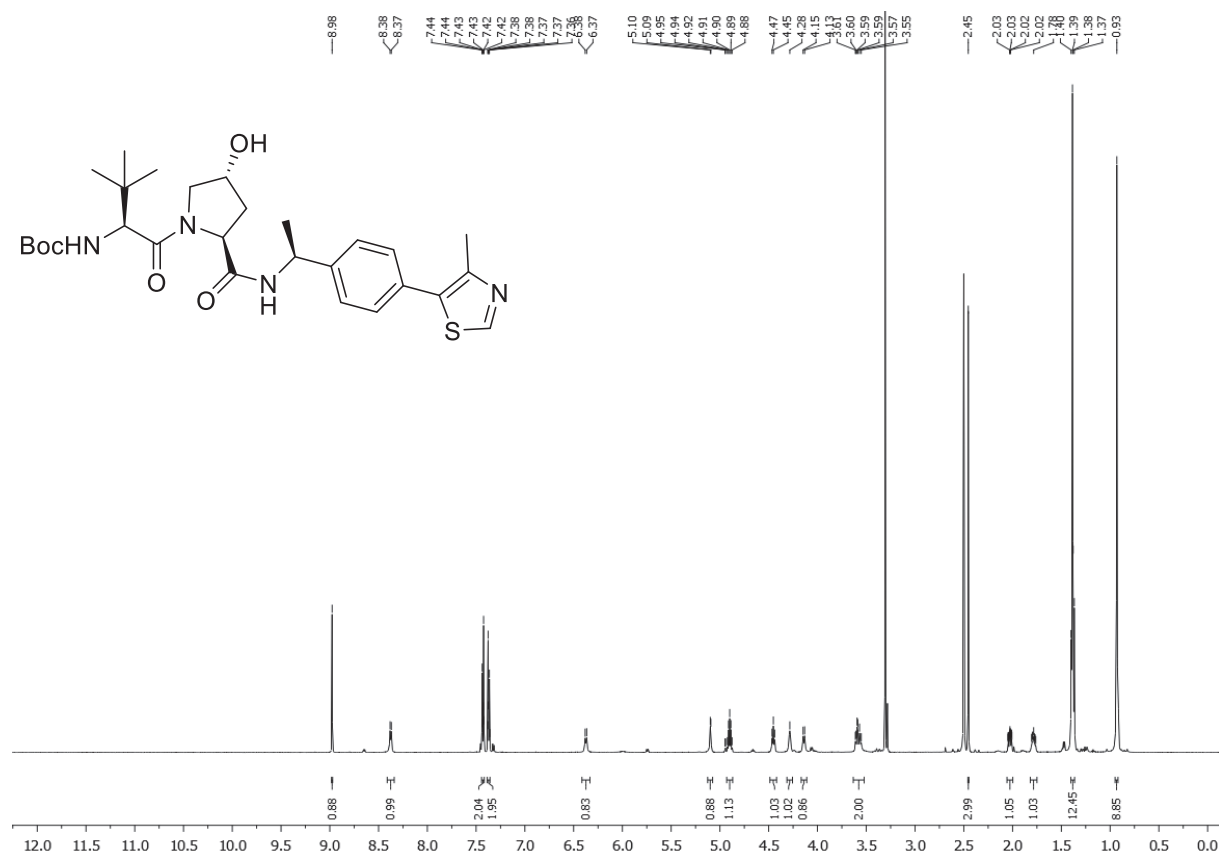
¹H NMR spectrum of **41** (500 MHz, DMSO-*d*₆)



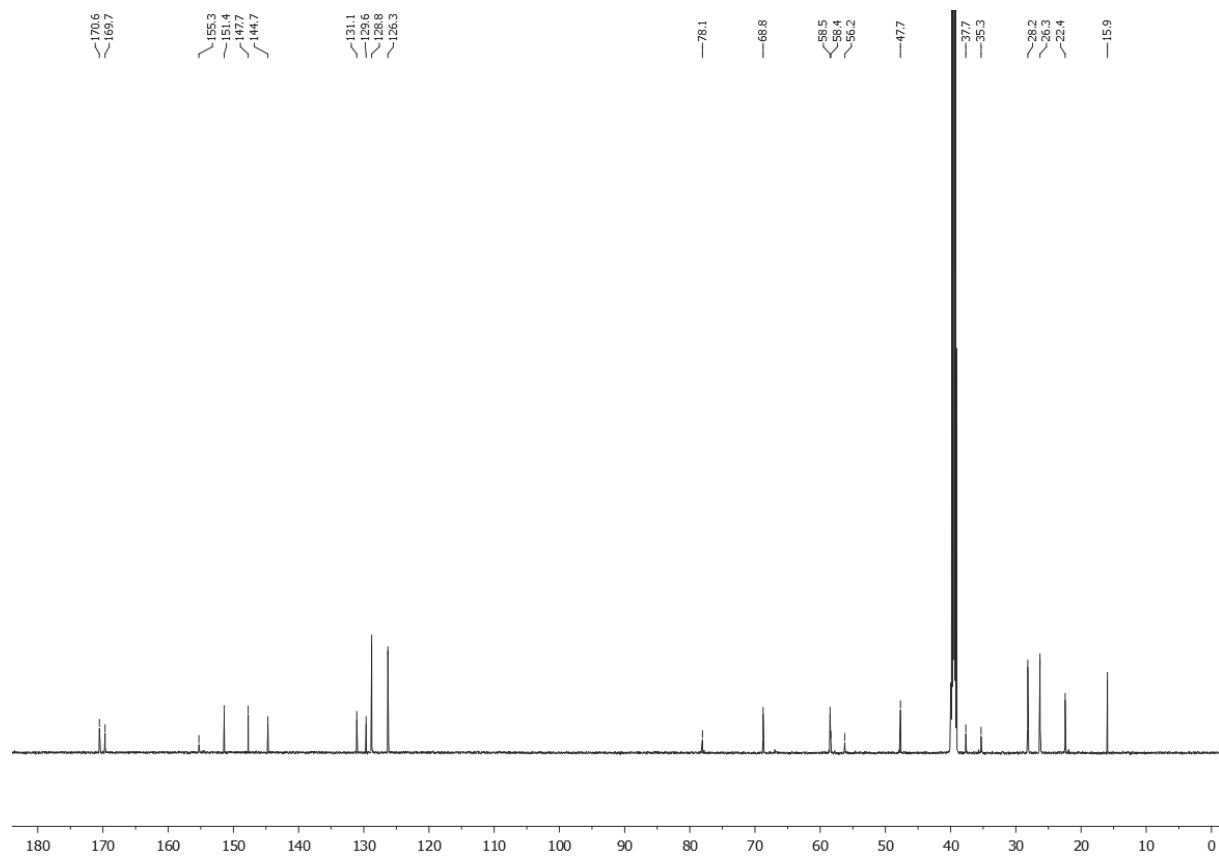
¹³C NMR spectrum of **41** (126 MHz, DMSO-*d*₆)



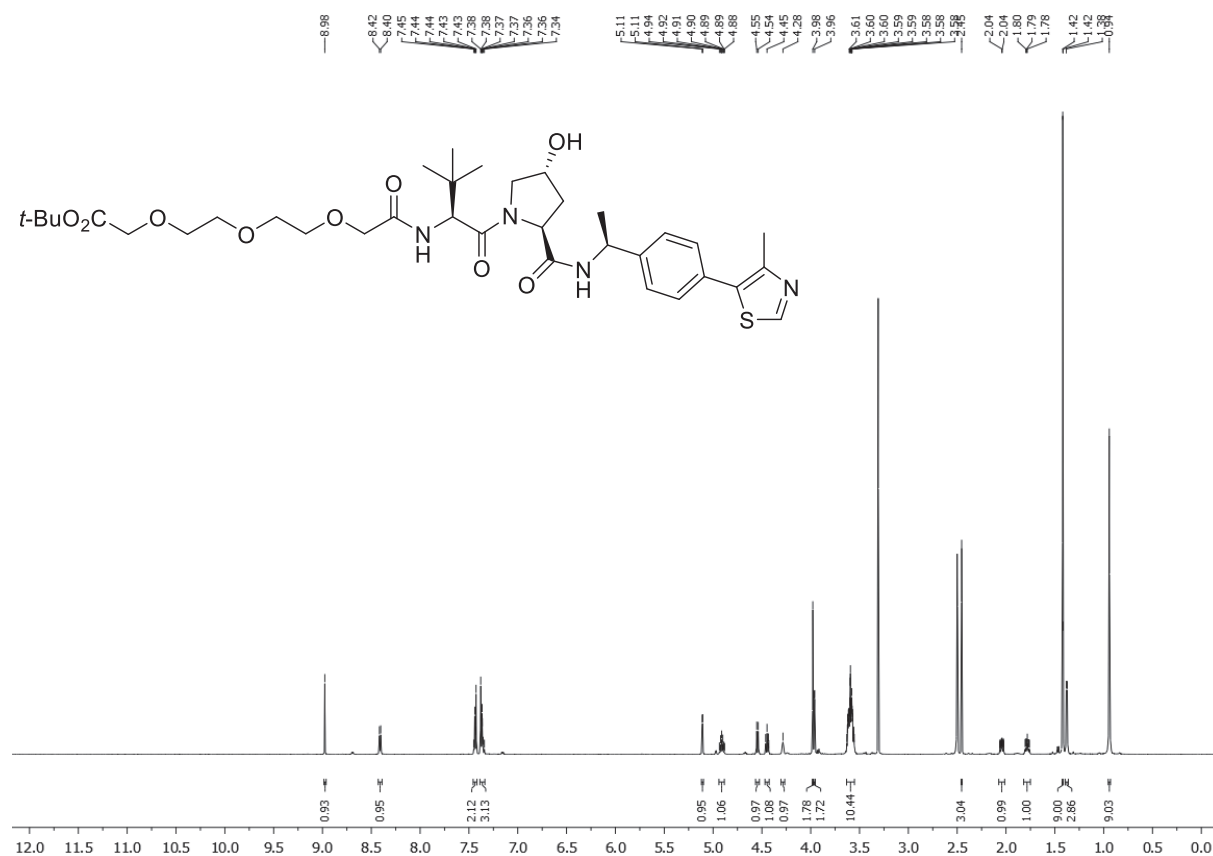
¹H NMR spectrum of **33** (600 MHz, DMSO-*d*₆)



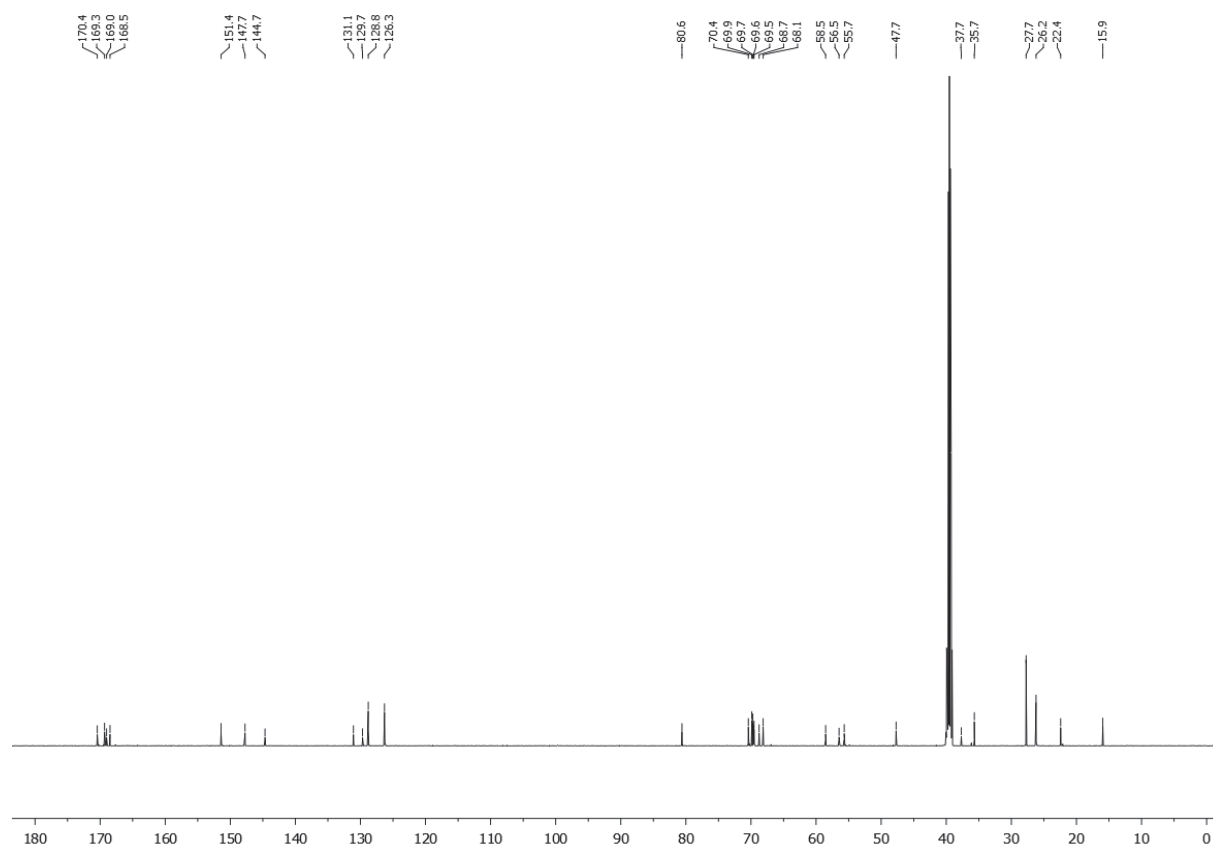
¹³C NMR spectrum of **33** (151 MHz, DMSO-*d*₆)



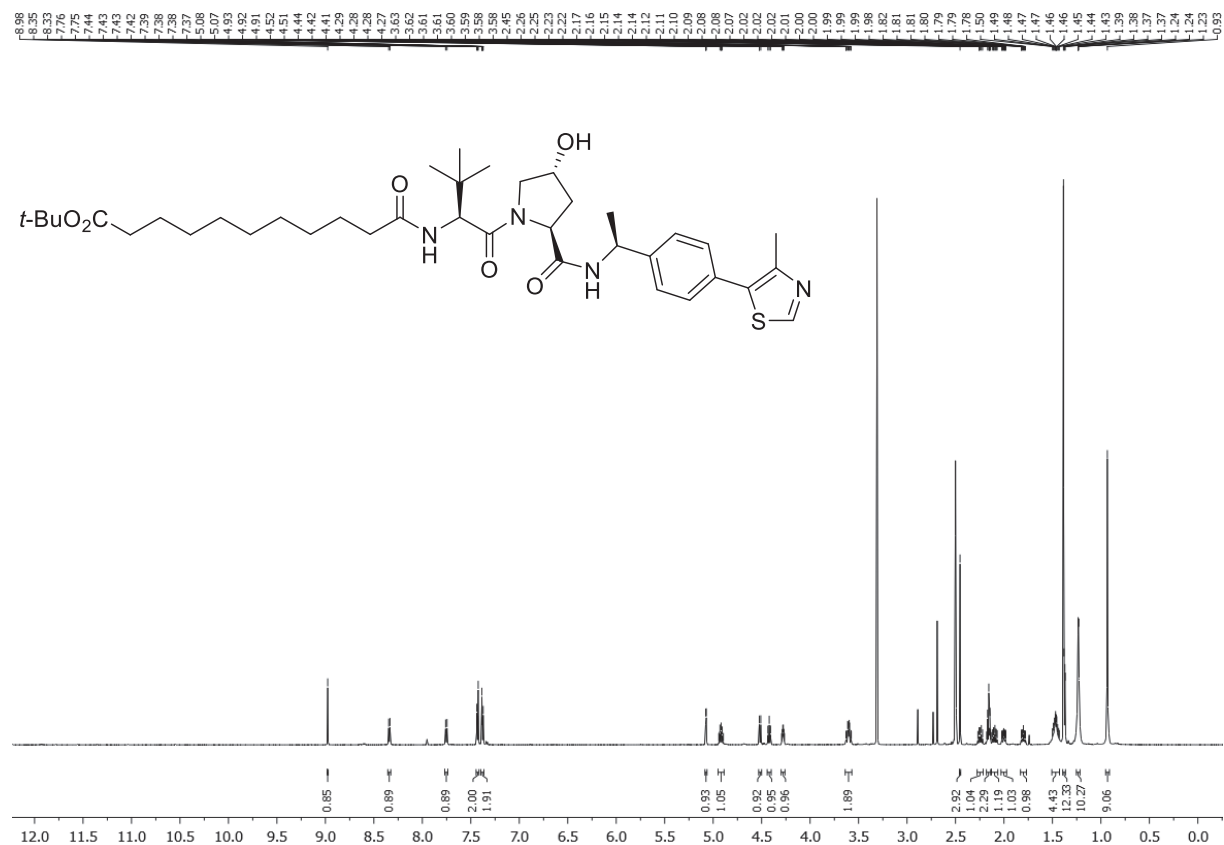
¹H NMR spectrum of **34** (600 MHz, DMSO-*d*₆)



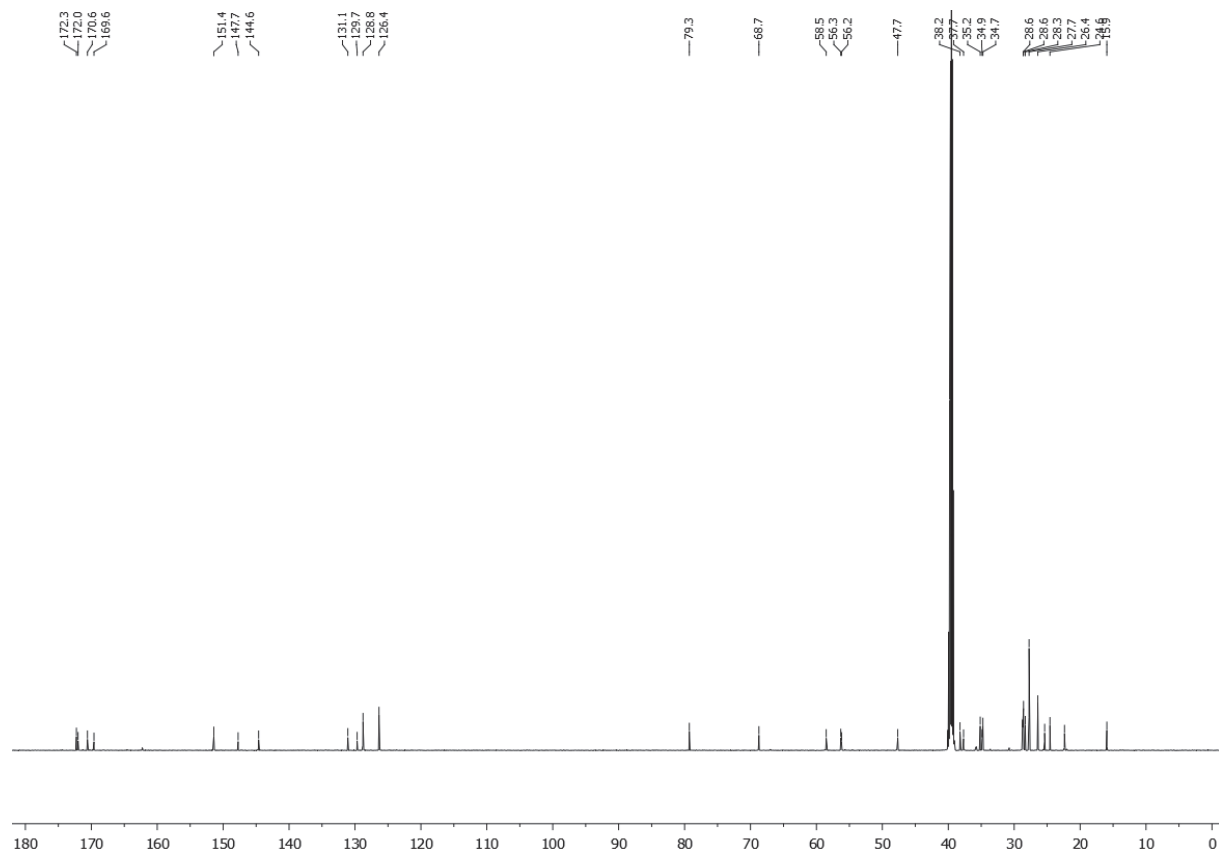
¹³C NMR spectrum of **34** (151 MHz, DMSO-*d*₆)



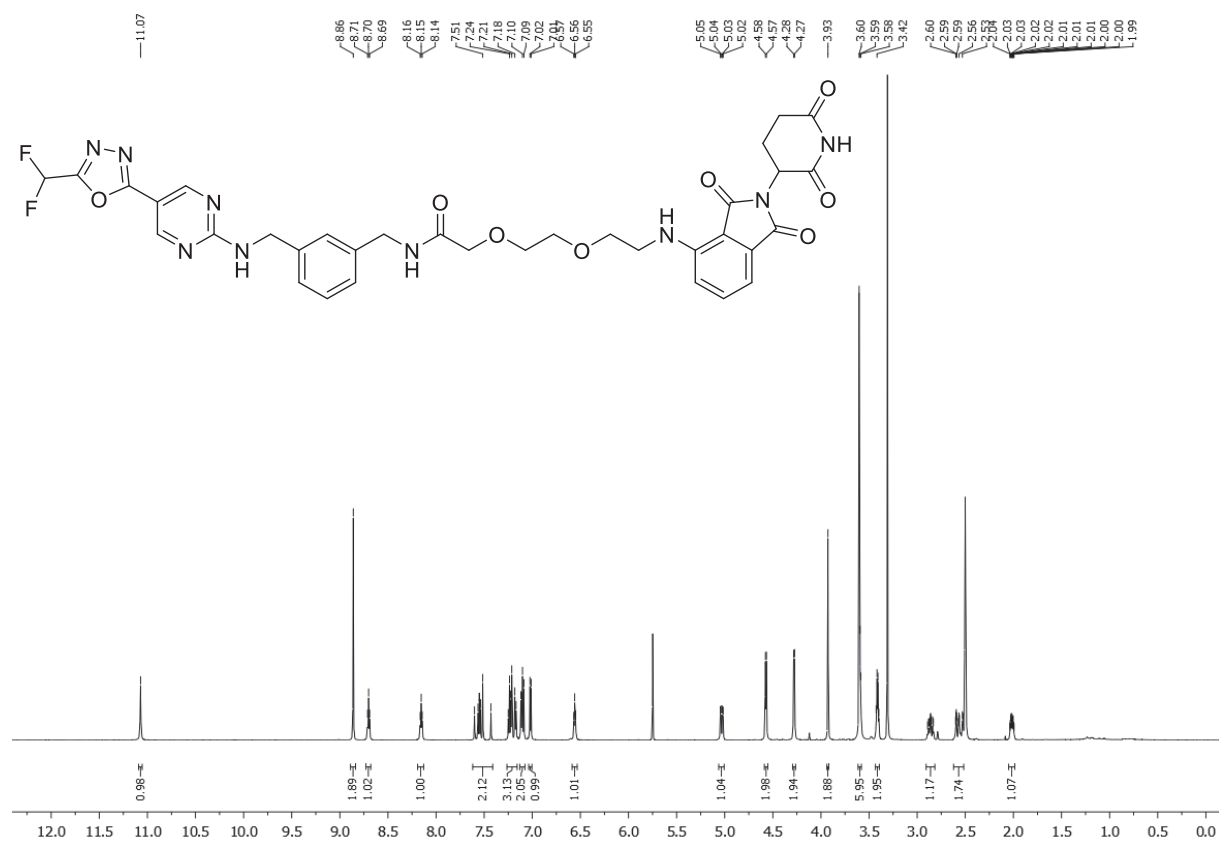
¹H NMR spectrum of **35 (600 MHz, DMSO-*d*₆)**



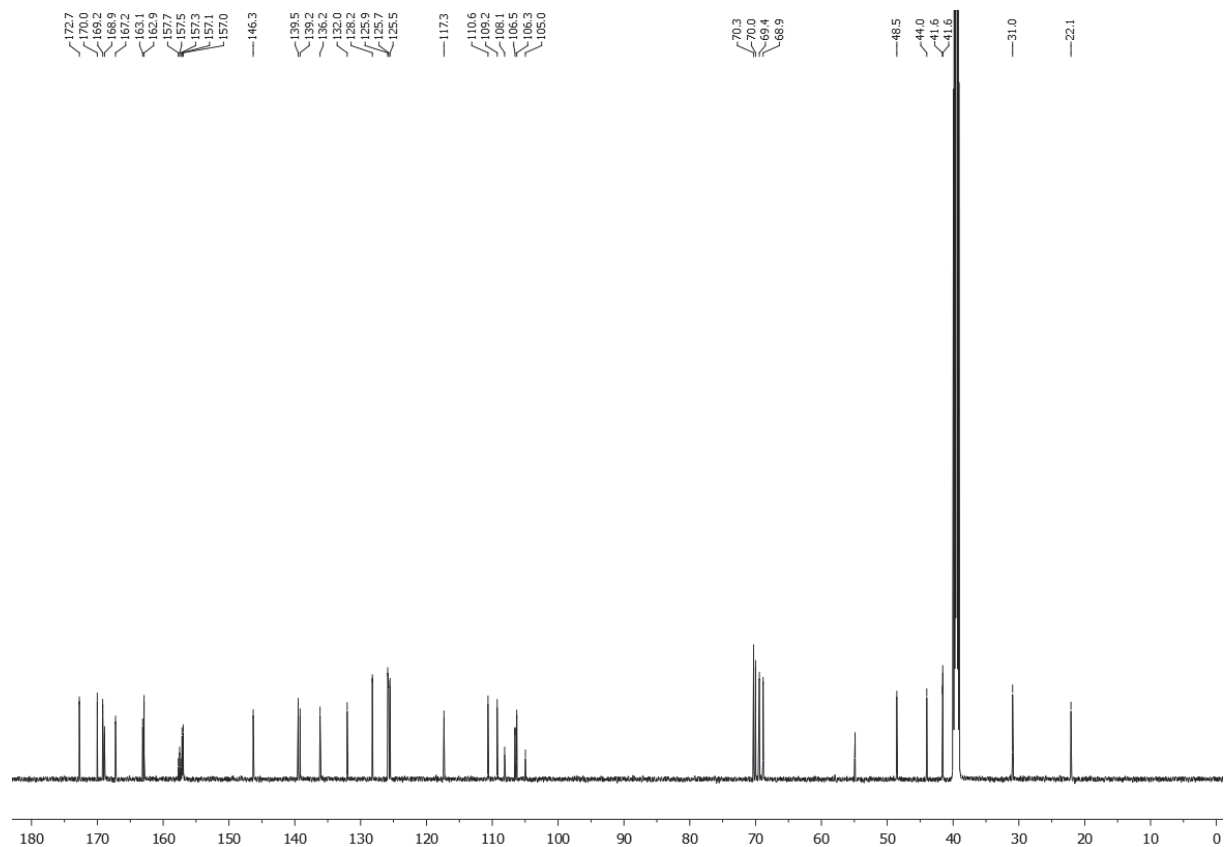
¹³C NMR spectrum of **35 (151 MHz, DMSO-*d*₆)**



¹H NMR spectrum of 1 (600 MHz, DMSO-*d*₆)



¹³C NMR spectrum of 1 (151 MHz, DMSO-*d*₆)



13C NMR spectrum (CDCl₃) of compound 10. The x-axis represents the chemical shift in ppm, ranging from 180 to 10. The spectrum shows several sharp peaks, with the most intense at 41.8 ppm. The following table lists the labeled peaks and their corresponding chemical shifts:

Chemical Shift (ppm)
172.8
172.0
170.0
168.3
167.3
163.1
162.9
157.7
157.5
157.3
157.1
157.0
146.4
139.9
139.2
135.2
132.2
138.2
125.8
125.7
125.4
117.1
110.3
109.0
108.1
106.5
106.3
105.0
48.5
44.0
41.9
41.8
35.3
31.0
28.6
28.6
28.4
26.2
25.2
22.1

Chemical structure of compound 10:

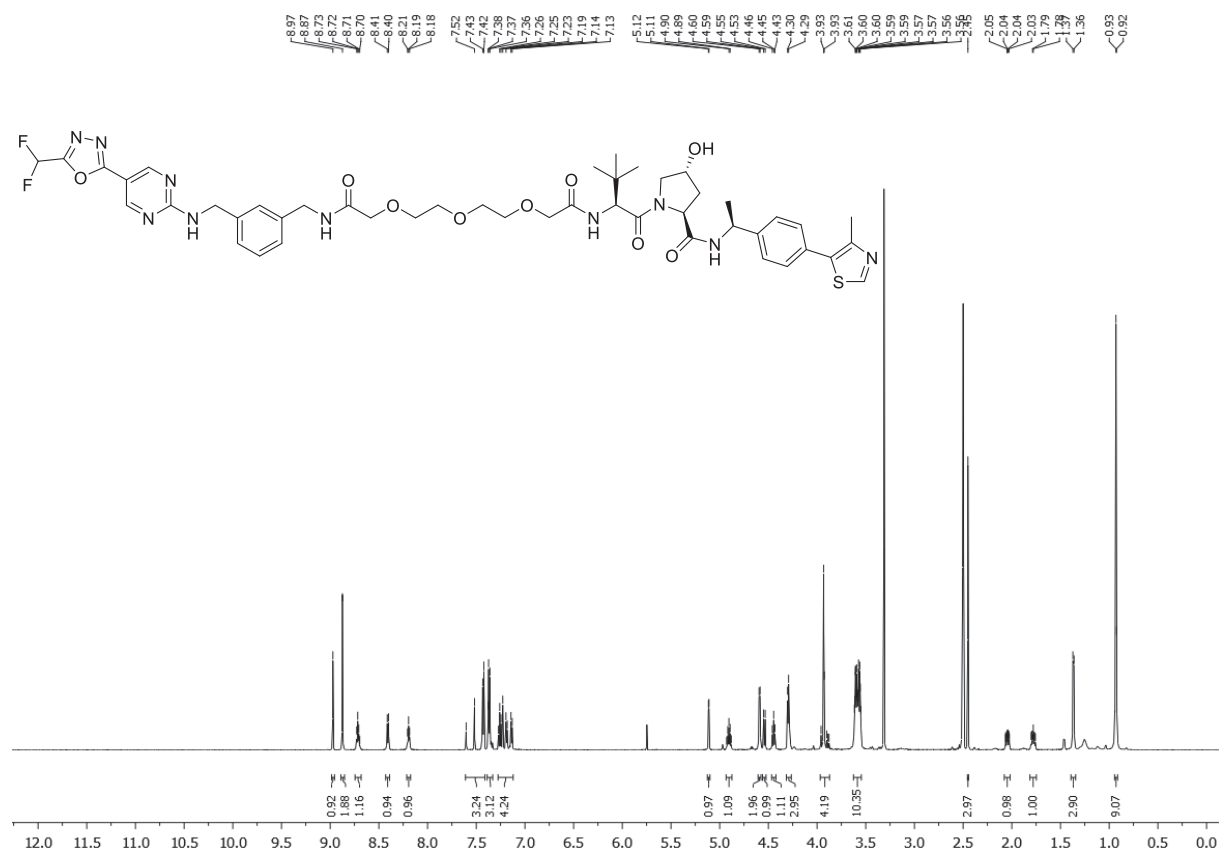
Fc1cc(F)nn1-c1nn(Cc2ccc(CNC(=O)COCCOCCNC3C(=O)c4ccccc4C3=O)cc2)c2

¹H NMR spectrum (CDCl₃):

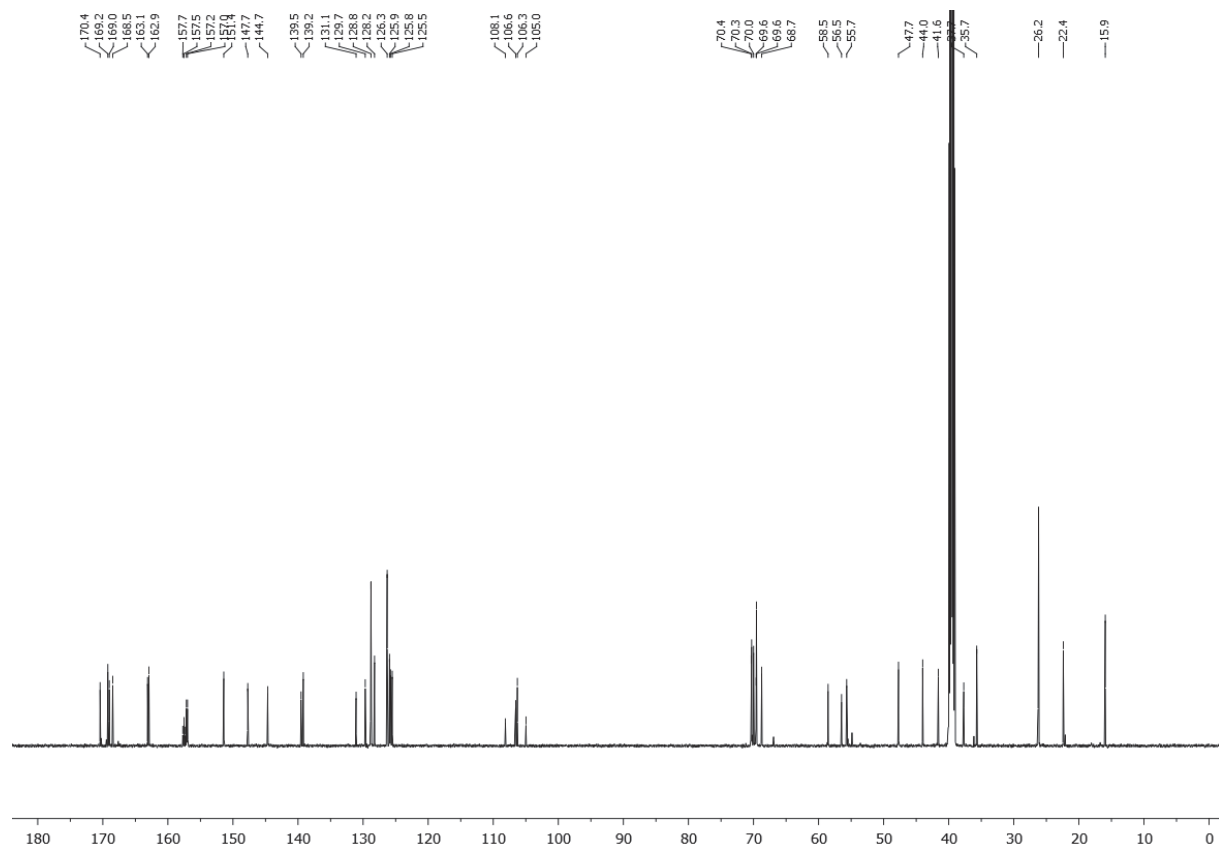
Chemical Shift (ppm)	Integration
11.07	1.03
8.69	1.83
8.68	1.04
8.67	1.00
8.13	2.17
8.12	2.05
8.11	2.02
7.90	1.00
7.89	1.00
7.55	1.00
7.54	1.00
7.51	1.00
7.43	1.00
7.25	1.00
7.24	1.00
7.19	1.00
7.18	1.00
7.10	1.00
7.09	1.00
7.03	1.00
6.57	1.00
6.56	1.00
6.55	1.00
5.75	1.00
5.05	1.00
5.04	1.00
5.03	1.00
5.02	1.00
4.56	1.00
4.55	1.00
4.27	1.00
4.26	1.00
3.93	1.00
3.91	1.00
3.90	1.00
3.89	1.00
3.43	1.00
3.42	1.00
3.41	1.00
3.40	1.00
2.89	1.06
2.88	1.06
2.87	1.06
2.86	1.06
2.84	1.06
2.83	1.06
2.60	2.02
2.59	2.07
2.57	2.02
2.56	2.02
2.55	6.35
2.54	2.06
2.52	2.06
2.51	2.06
2.04	1.10
2.03	1.81
2.02	1.81
2.01	1.09
2.00	1.09
1.99	1.09
1.25	1.25
1.24	1.24
1.23	1.24
1.11	1.11
1.10	1.10
1.09	1.09
1.08	1.08
1.07	1.07
0.85	0.85

172.7
170.0
169.1
168.9
167.2
163.1
162.9
157.7
157.3
157.1
157.0
146.3
137.9
137.7
136.1
132.0
127.2
127.0
117.3
110.6
109.2
108.1
106.5
106.3
105.0
70.3
70.0
69.4
68.8
48.5
43.8
41.6
41.4
30.9
22.1

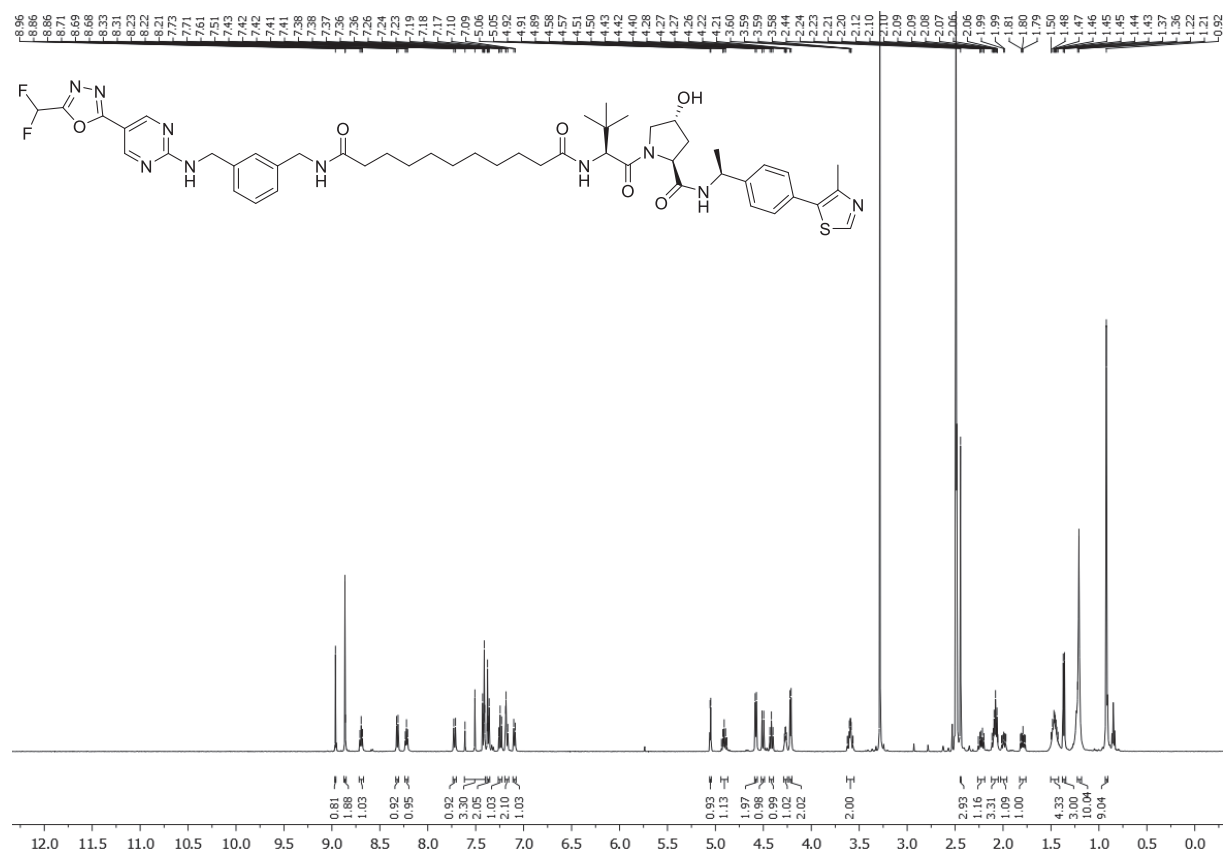
¹H NMR spectrum of 4 (600 MHz, DMSO-*d*₆)



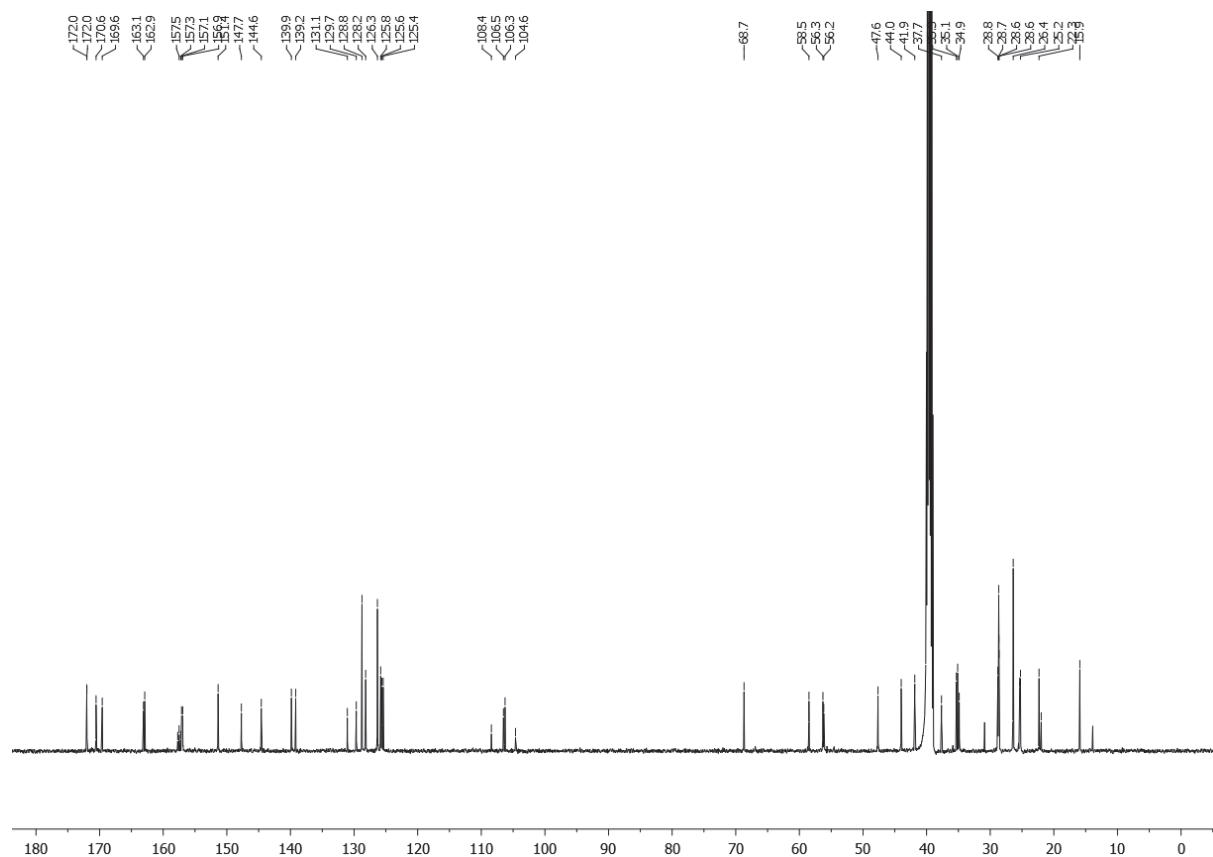
¹³C NMR spectrum of 4 (151 MHz, DMSO-*d*₆)



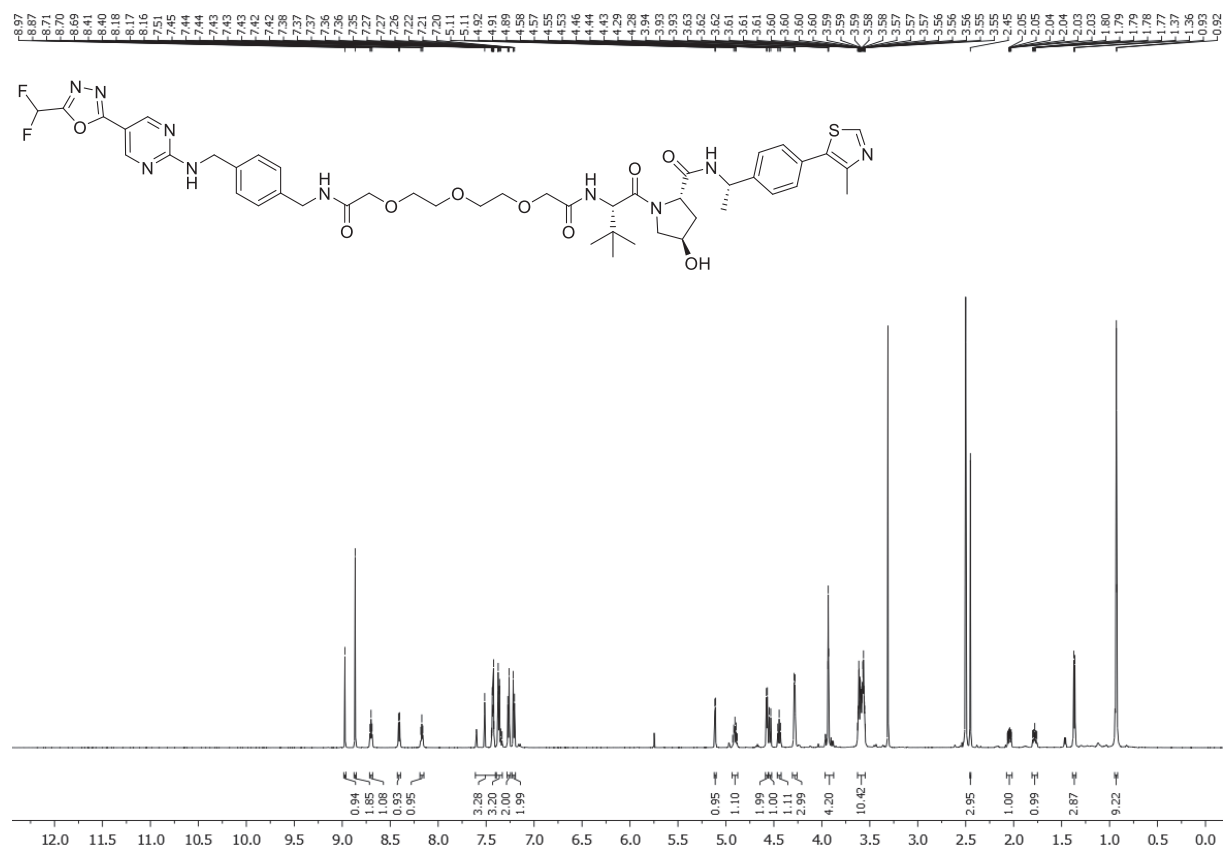
¹H NMR spectrum of **5 (500 MHz, DMSO-*d*₆)**



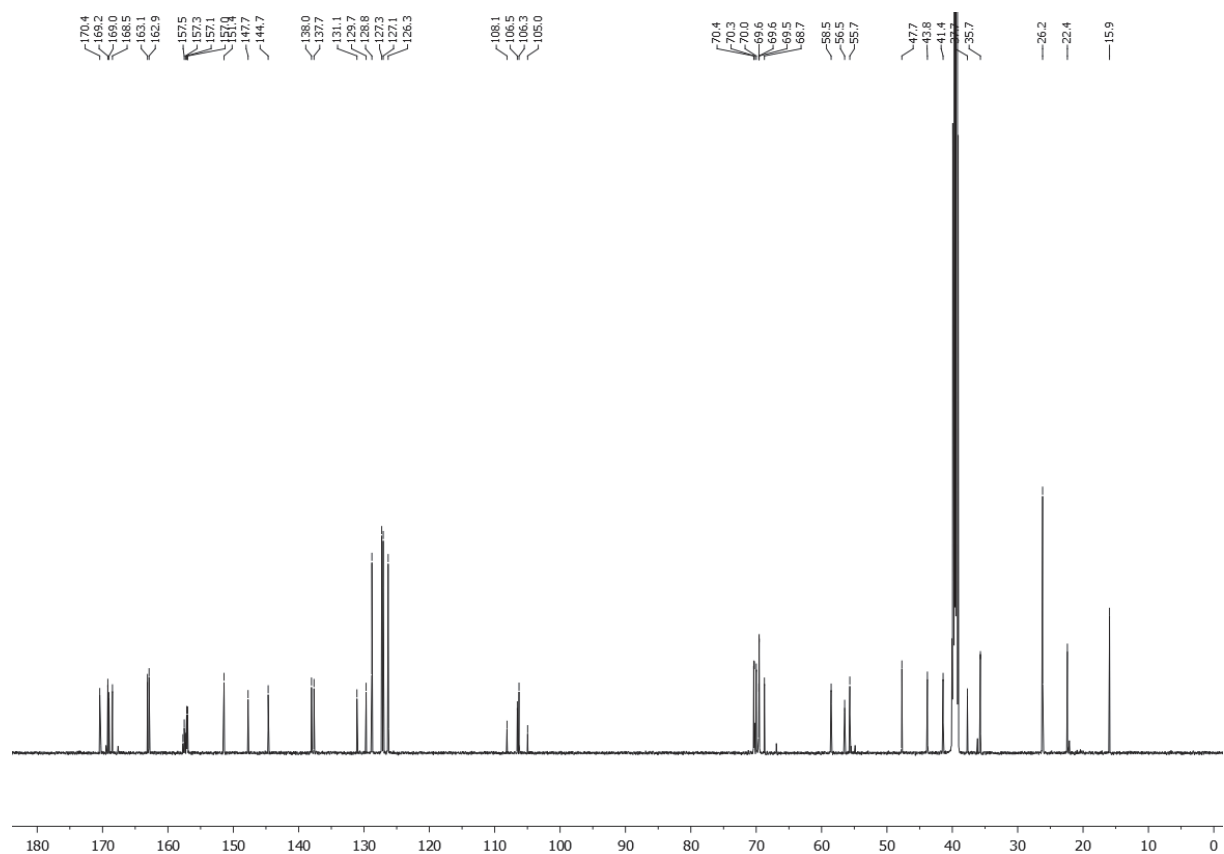
¹³C NMR spectrum of **5 (126 MHz, DMSO-*d*₆)**



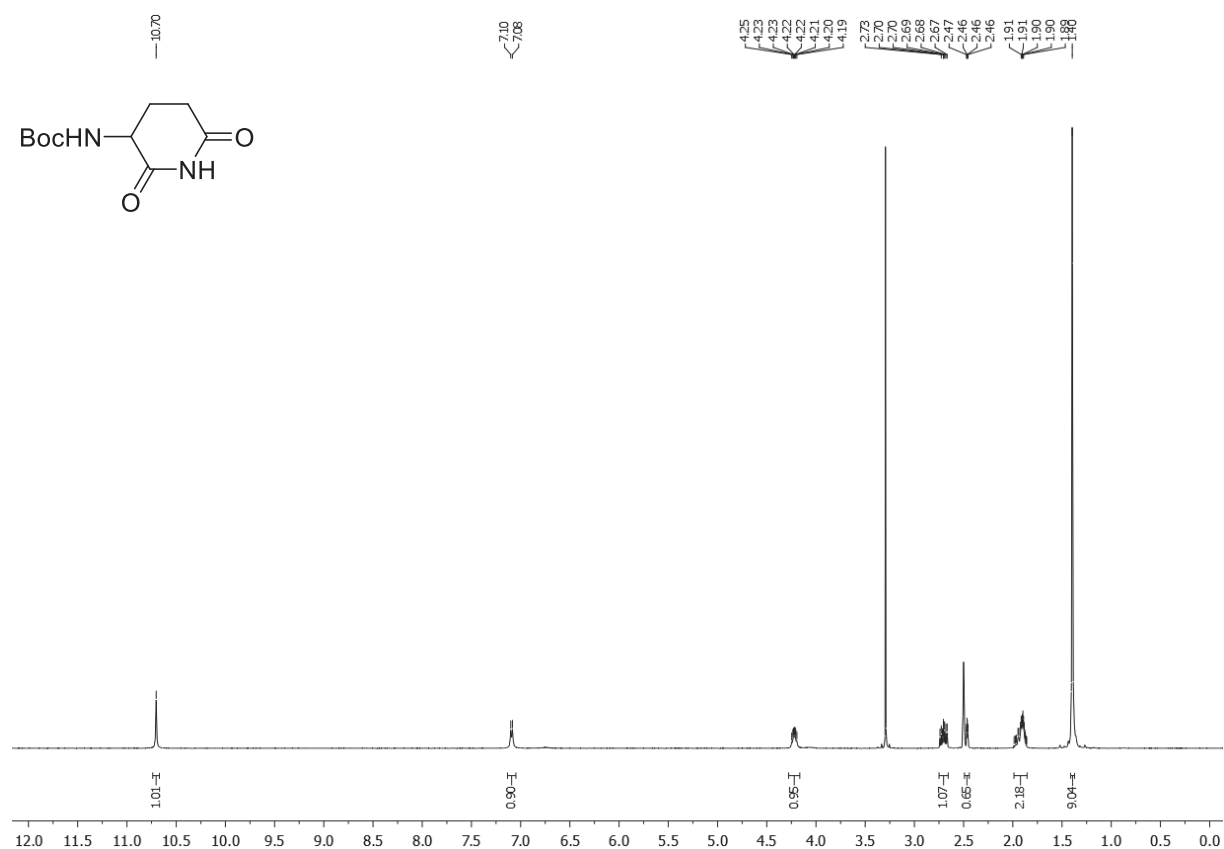
¹H NMR spectrum of 6 (600 MHz, DMSO-*d*₆)



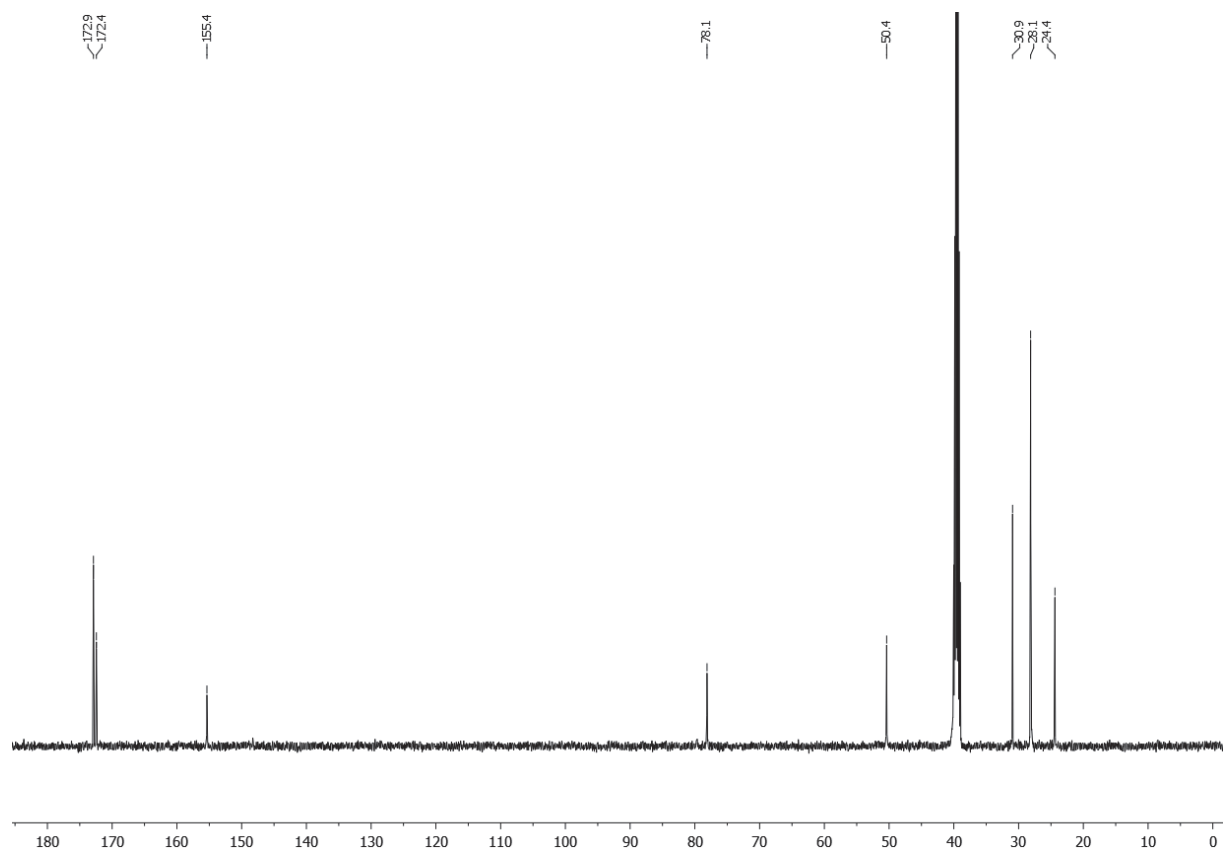
¹³C NMR spectrum of 6 (151 MHz, DMSO-*d*₆)



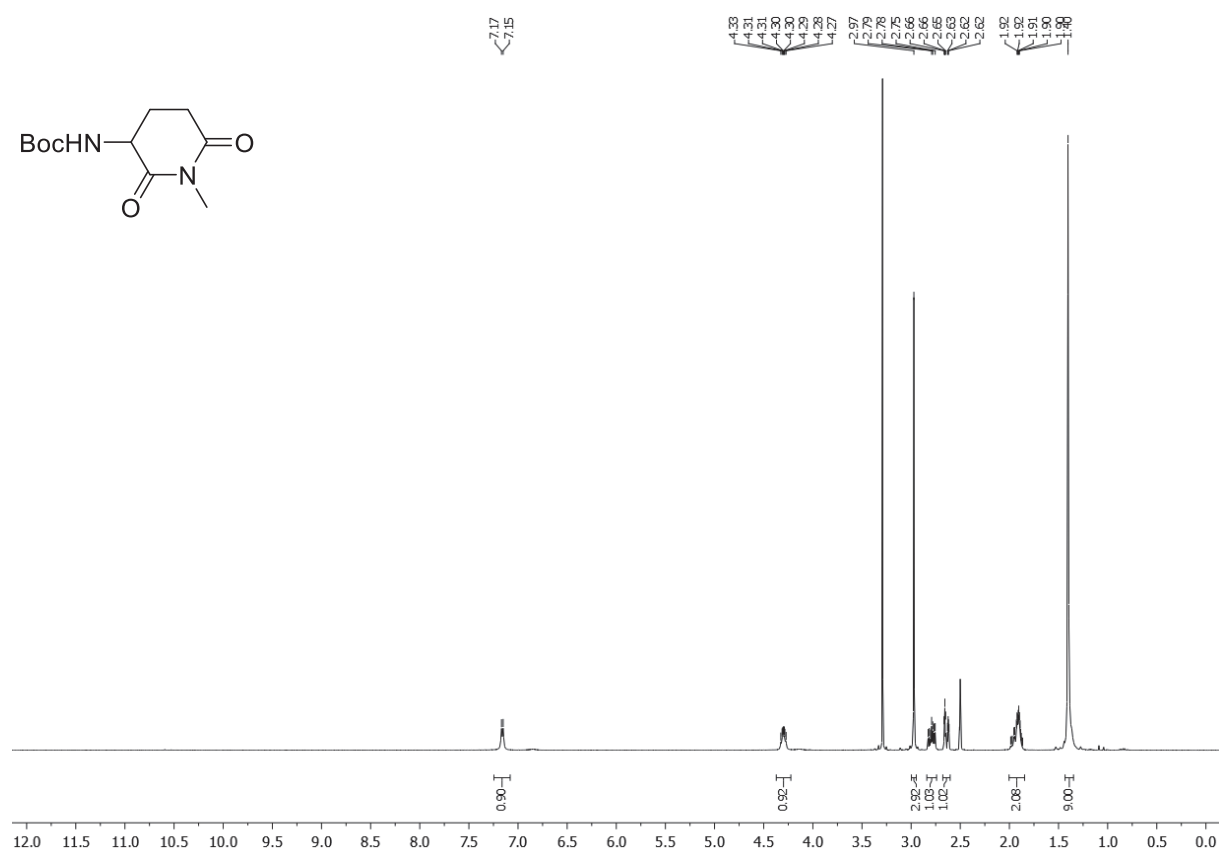
^1H NMR spectrum of **42** (500 MHz, $\text{DMSO-}d_6$)



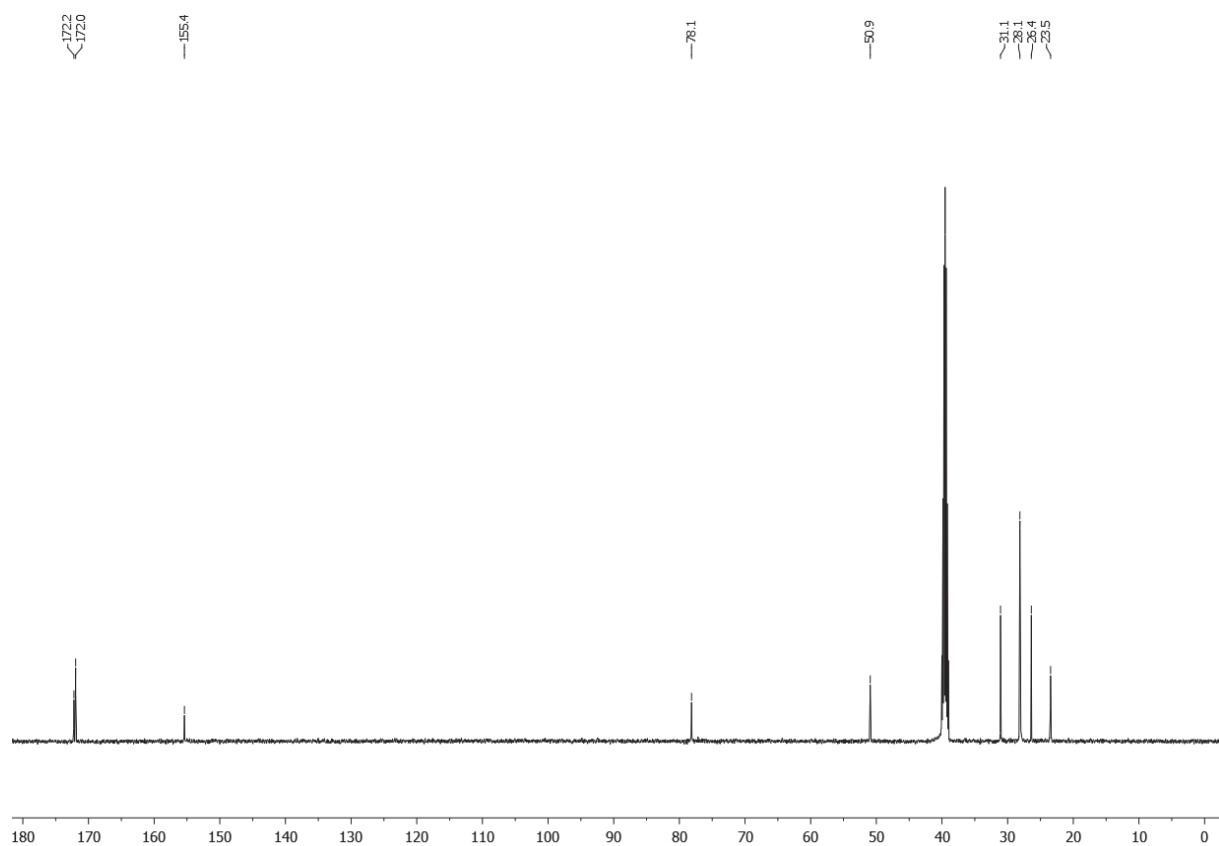
^{13}C NMR spectrum of **42** (126 MHz, $\text{DMSO-}d_6$)



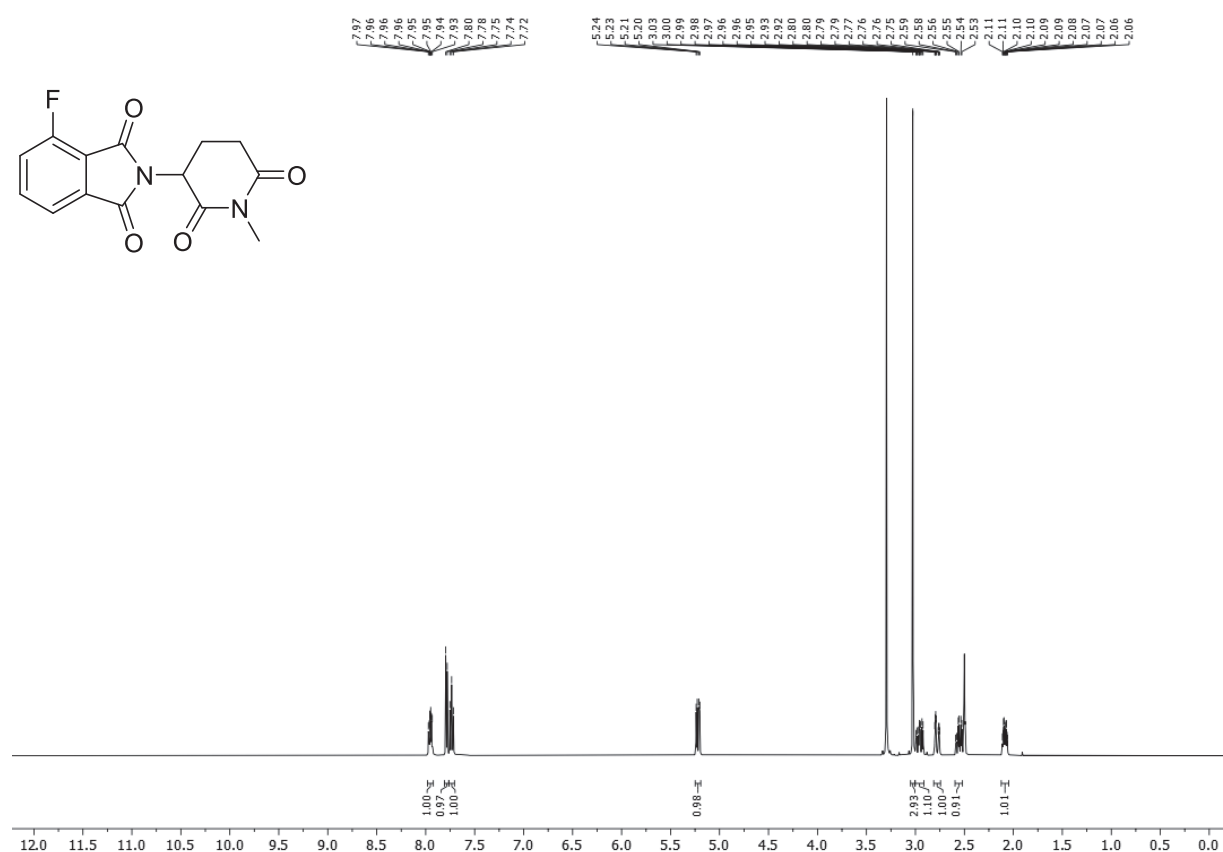
¹H NMR spectrum of **43 (500 MHz, DMSO-*d*₆)**



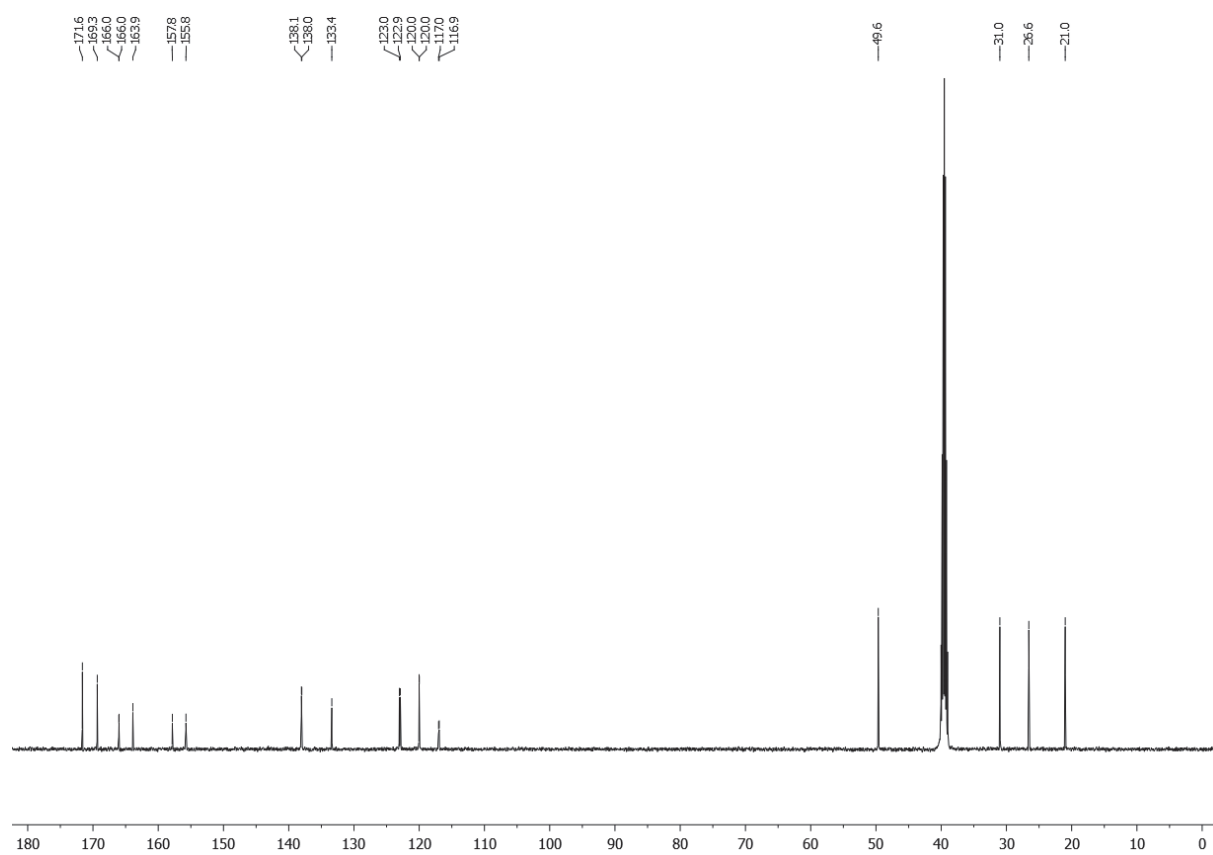
¹³C NMR spectrum of **43 (126 MHz, DMSO-*d*₆)**



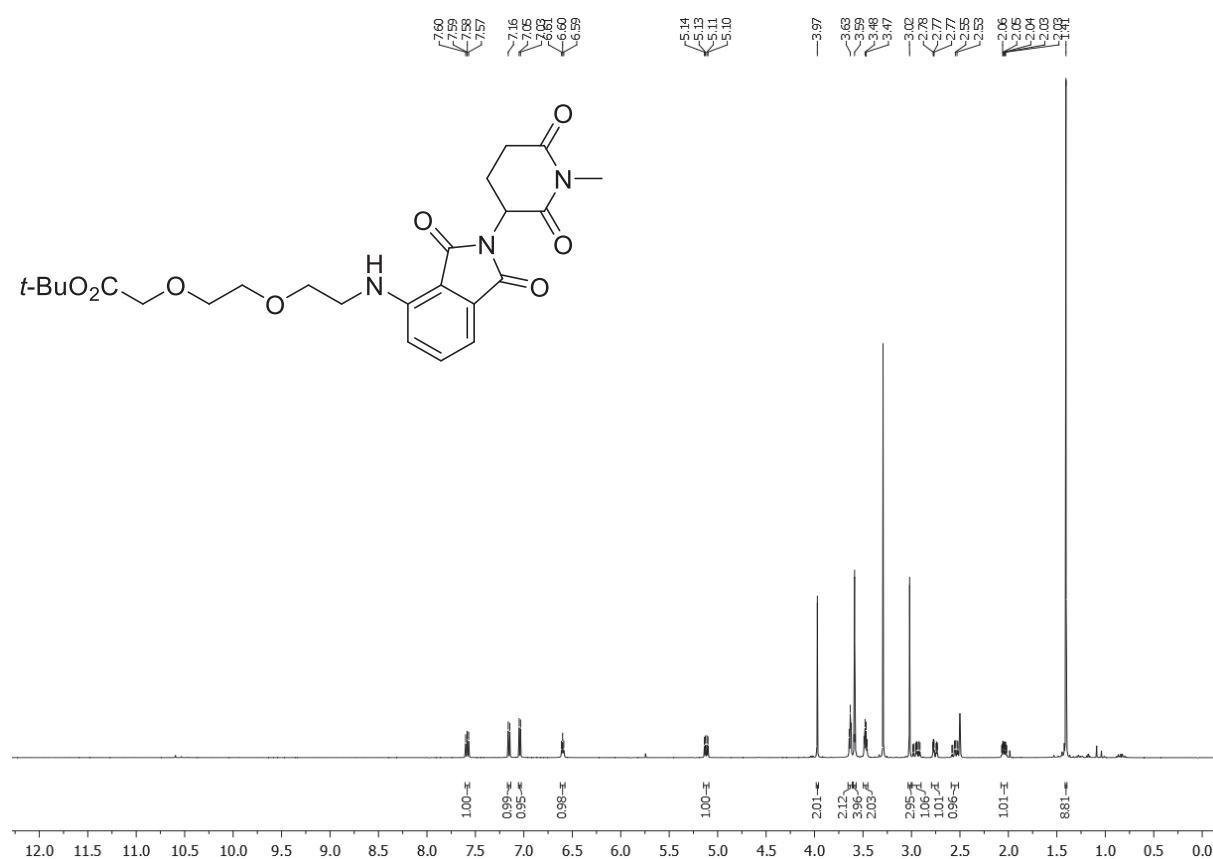
¹H NMR spectrum of 44 (500 MHz, DMSO-*d*₆)



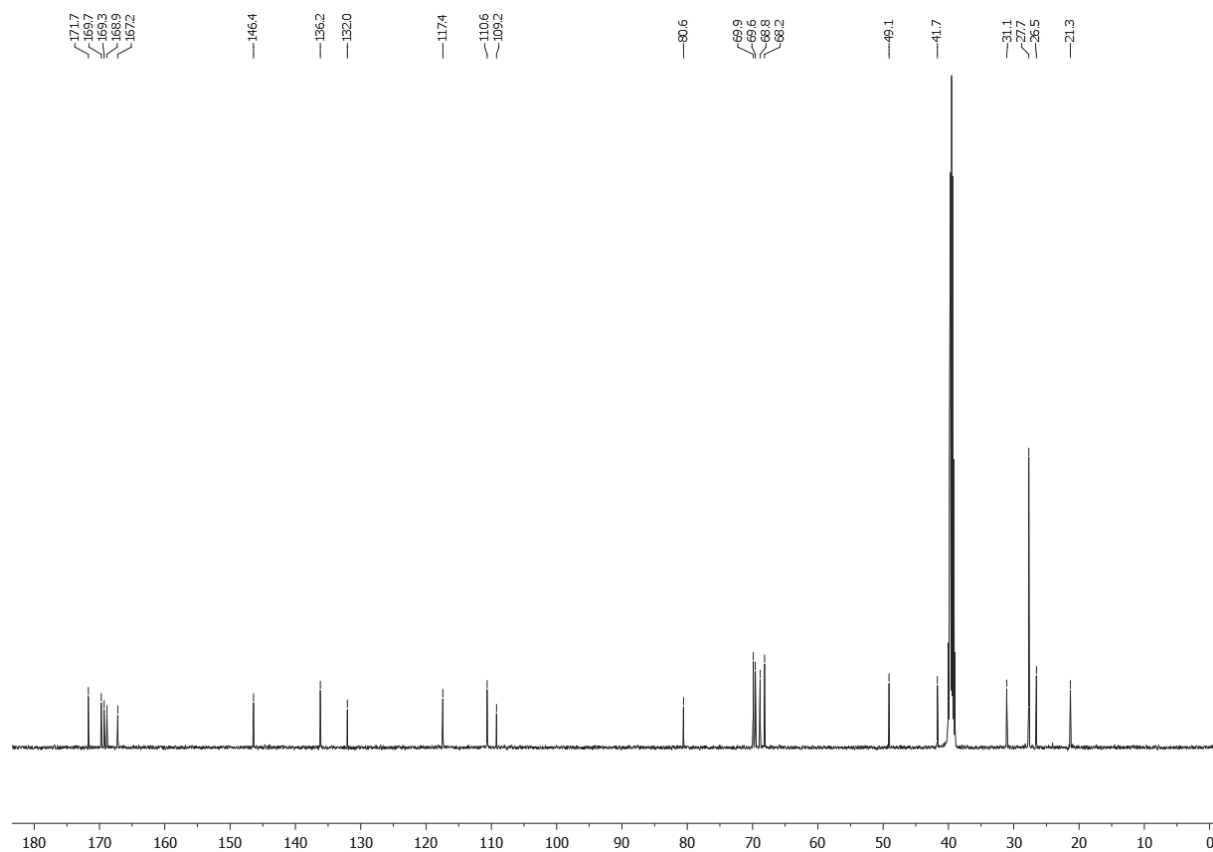
¹³C NMR spectrum of 44 (126 MHz, DMSO-*d*₆)



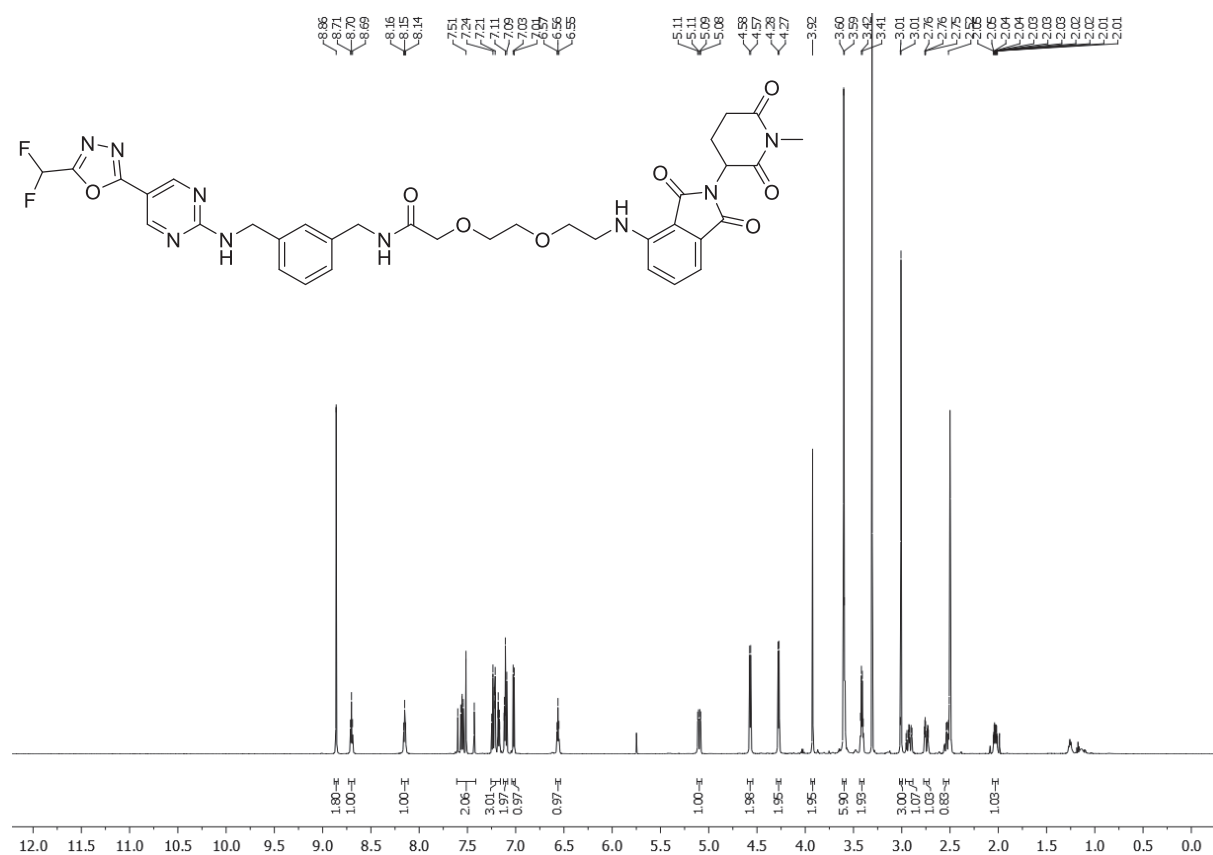
¹H NMR spectrum of **45** (500 MHz, DMSO-*d*₆)



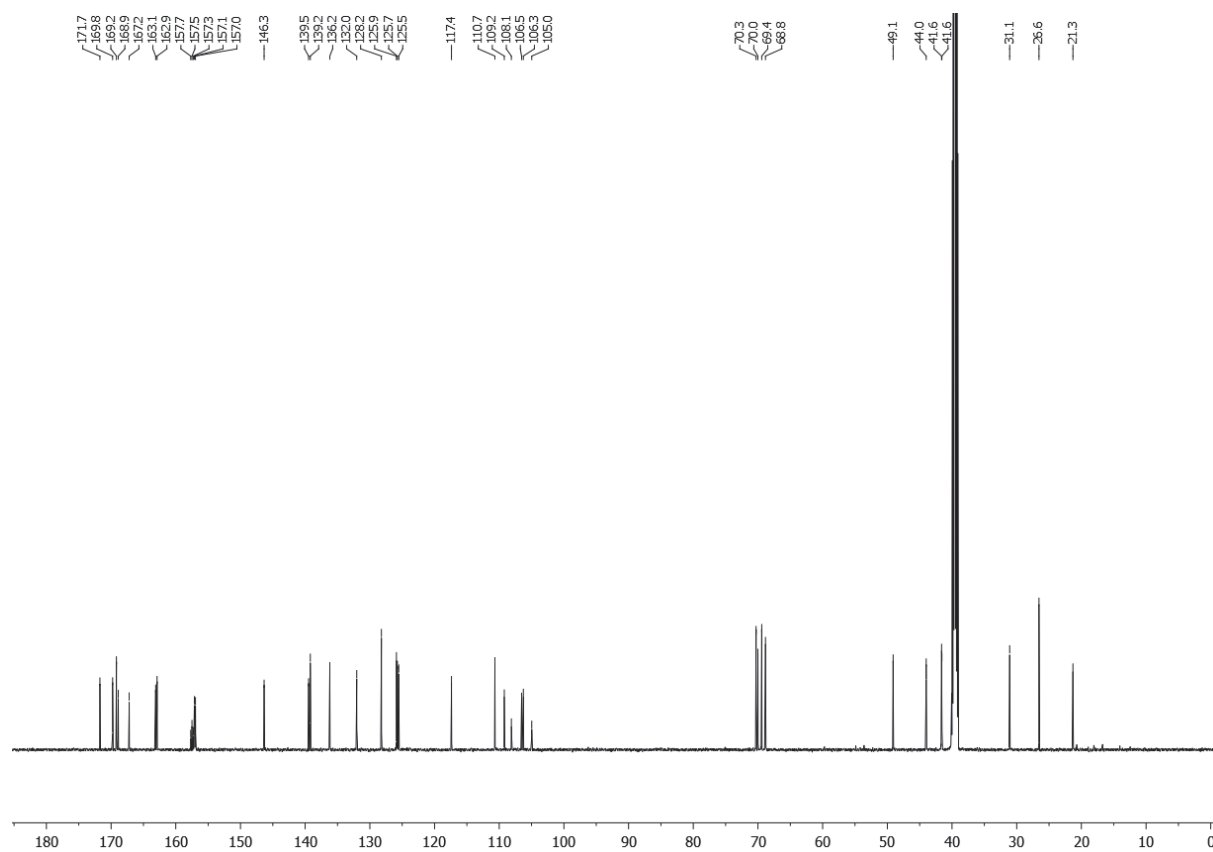
¹³C NMR spectrum of **45** (126 MHz, DMSO-*d*₆)



¹H NMR spectrum of 1(-) (600 MHz, DMSO-*d*₆)



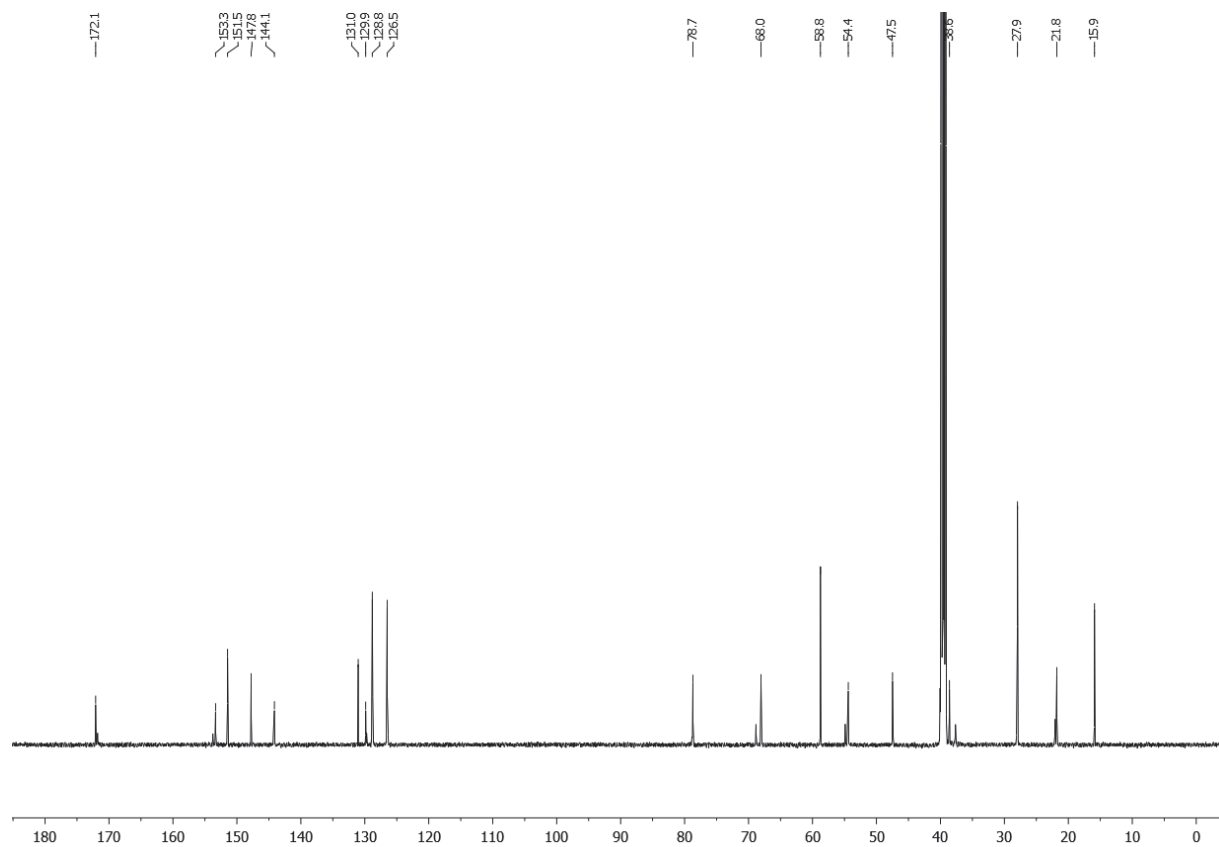
¹³C NMR spectrum of 1(-) (151 MHz, DMSO-*d*₆)



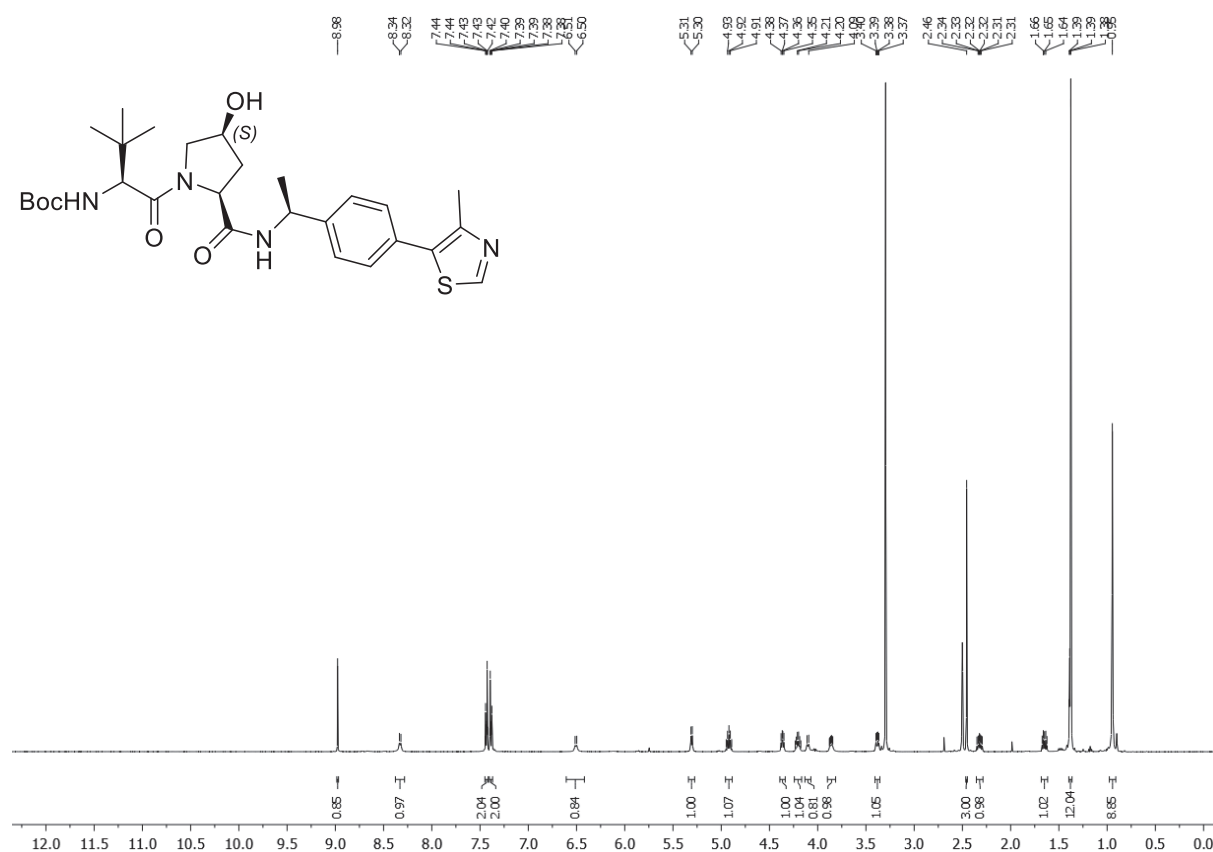
¹H NMR spectrum of **46** (600 MHz, DMSO-*d*₆)



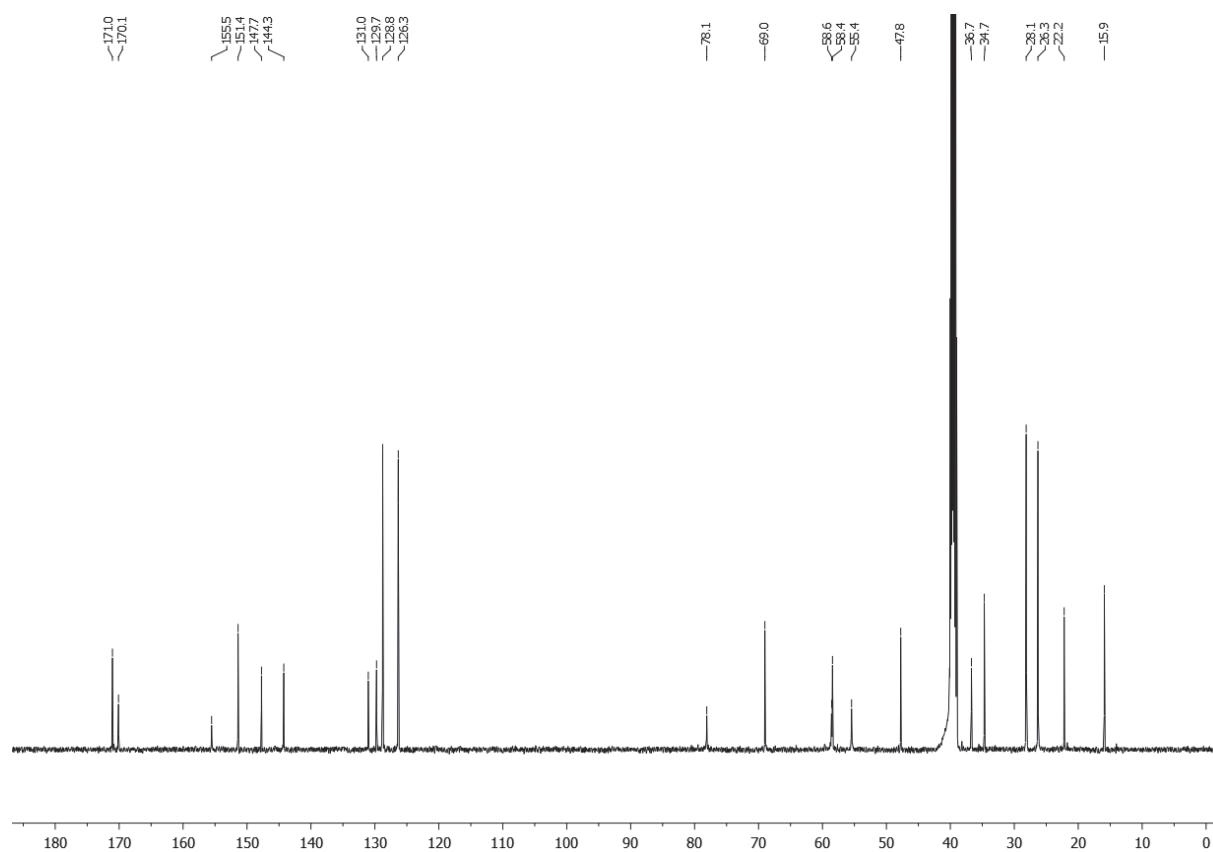
¹³C NMR spectrum of **46** (151 MHz, DMSO-*d*₆)



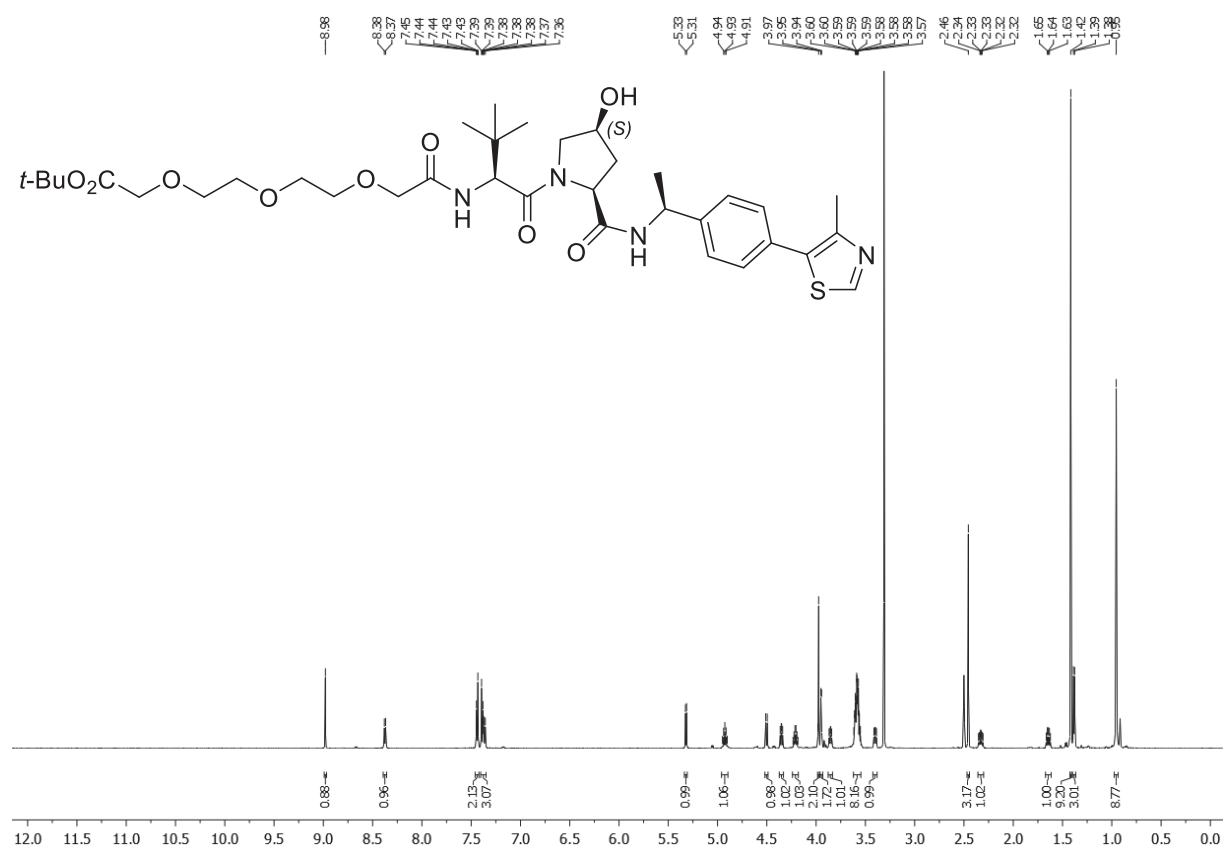
¹H NMR spectrum of 47 (500 MHz, DMSO-*d*₆)



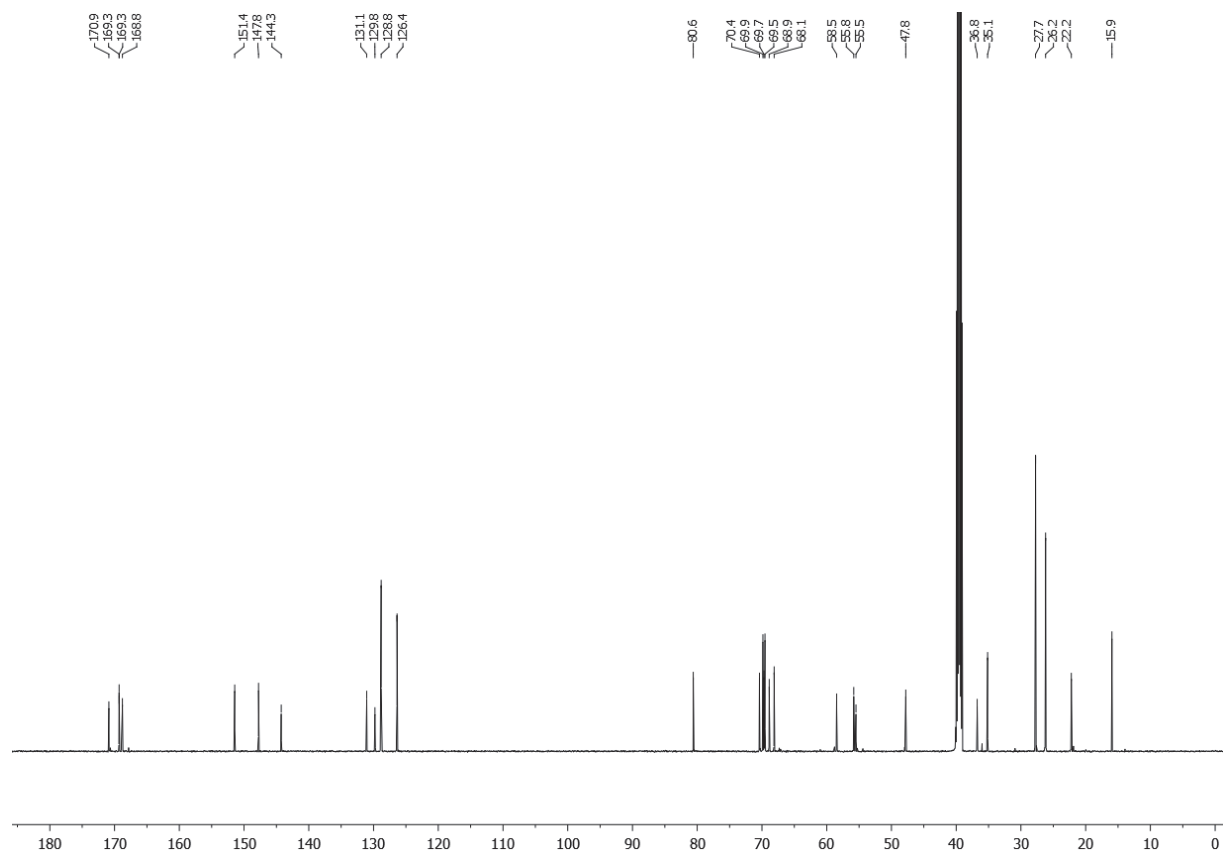
¹³C NMR spectrum of 47 (126 MHz, DMSO-*d*₆)



¹H NMR spectrum of **48** (600 MHz, DMSO-*d*₆)



¹³C NMR spectrum of **48** (151 MHz, DMSO-*d*₆)



[illegible]

Mass spectrum of compound 10. The x-axis represents the mass-to-charge ratio (m/z) from 180 to 10, and the y-axis represents relative intensity from 0 to 100. The base peak is at m/z 41.6. Other labeled peaks include:

m/z	Relative Intensity (approx.)
170.9	5
169.3	5
168.8	5
168.1	5
163.1	5
162.9	5
157.7	10
157.5	10
157.2	10
151.9	10
147.8	10
144.3	10
139.5	10
139.2	10
131.0	10
129.8	10
128.8	10
128.2	10
125.4	10
125.8	10
125.5	10
108.1	5
106.6	5
105.3	5
105.0	5
70.3	10
70.3	10
70.0	10
68.6	10
68.5	10
68.9	10
58.5	10
58.8	10
55.5	10
47.8	10
44.0	10
41.6	100
36.8	10
35.2	10
26.2	10
22.2	10
15.9	10

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Appendix II. Publication II: Structure–Stability Relationship of NLRP3 Inflammasome-Inhibiting Sulfonylureas

The following pages include the article “Structure–Stability Relationship of NLRP3 Inflammasome-Inhibiting Sulfonylureas” as it was published in ACS Omega by ACS Publications.

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Structure–Stability Relationship of NLRP3 Inflammasome-Inhibiting Sulfonylureas

Tim Keuler, Dominic Ferber, Michael Marleaux, Matthias Geyer, and Michael Gütschow*

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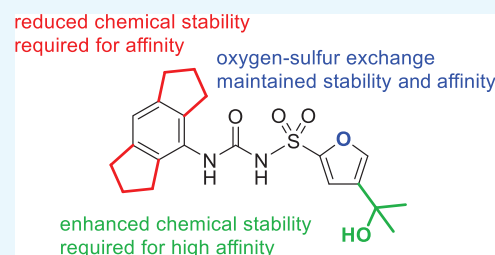


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Supporting Information

ABSTRACT: In recent drug development efforts, particular emphasis has been devoted to the chemical interference with the NLRP3 inflammasome. A series of 12 tailored sulfonylureas was designed, prepared through convergent syntheses with a final sodium hydride-promoted reaction of isocyanates and sulfonamides, and subjected to a systematic, high-performance liquid chromatography-based survey of the chemical stability, a critical issue of sulfonylureas in terms of preparation, storage, and application. NLRP3 binding was determined by surface plasmon resonance spectroscopy. Sulfonylurea 2 was identified to be equipotent and similarly stable compared to the prototypical NLRP3 inhibitor MCC950.



INTRODUCTION

Sulfonylureas had been the first-line drugs for diabetics for almost 6 decades. Their molecular targets, sulfonylurea receptors, are subunits of the octameric ATP-sensitive potassium channels. Upon sulfonylurea binding, the potassium efflux is decreased and the voltage-gated calcium channels are activated, ultimately leading to insulin secretion.¹ In 2001, an established antidiabetic drug, glibenclamide, was observed to inhibit the formation of mature interleukin-1 β .² Sulfonylureas that affect the accompanied cellular response have been designated cytokine release inhibitory drugs (CRIDs).² The most prominent representative of this class of compounds,^{3,4} the diaryl sulfonylurea derivative MCC950 (1, Table 1), is also referred to as CRID3 or CP-456,773. Some of its relatives, but not MCC950 itself, were able to stimulate insulin secretion.⁵ The anti-inflammatory activity of MCC950 was attributed to the inhibition of the NLRP3 inflammasome.⁶ This finding paved the way for further studies on the specific interaction of the NLRP3 inflammasome with low-molecular-weight, drug-like compounds, which proved of great value as pharmacological tool compounds^{7,8} and progressed into clinical trials.⁹

NLRP3 is a tripartite inflammasome sensor protein.¹⁰ Its activation can be ubiquitously triggered by damage- or pathogen-associated molecular patterns, which leads to the oligomerization of NLRP3, followed by the recruitment of the adaptor protein ASC and pro-caspase-1 to an intracellular supramolecular complex. The assembly of the inflammasome platform induces the autoactivation of caspase-1. Acting as an effector protein, it cleaves pro-interleukin-1 β and pro-interleukin-18 to the mature cytokines and gasdermin-D to its N- and C-terminal domains. Subsequently, the gasdermin-D N-terminal domain forms membrane pores resulting in pyroptosis and the secretion of the pro-inflammatory cytokines.¹¹ NLRP3 has attracted great attention as a major therapeutic target and a key player in a variety of pathologies,

for example, rheumatoid arthritis, osteoarthritis, Crohn's disease, gout, coronary artery diseases, hepatic and pulmonary fibroses, as well as nonalcoholic steatohepatitis.^{9,12}

The molecular interaction of MCC950 and NLRP3 involves a high-affinity, noncovalent binding of the inhibitor to the Walker B ATP-hydrolysis motif of the NACHT domain,⁷ converting the NLRP3 "open" structure into a "closed" inactive conformation.^{7,13,14} Thus, NLRP3 inhibitors of this chemotype are thought to act as interdomain glues to lock the protein in an inactive conformation.¹⁴

The extraordinary potency of MCC950 with an IC₅₀ value of 7.5 nM was demonstrated in a cell-based interleukin-1 β assay.⁶ Pharmacokinetic studies showed the oral bioavailability and a plasma half-life of 3.27 h.⁶ MCC950 metabolites exhibited a strongly reduced inhibitory activity.¹⁵

Despite the resurgence of sulfonylureas as NLRP3-targeting drug candidates, a systematic study on their chemical stability has not been conducted yet. We performed a positional scanning with respect to the substituents at the sulfur and the terminal nitrogen of the central sulfonylurea core. Whereas the conventional antidiabetic sulfonylureas comprise *N*-alkyl, *S*-aryl derivatives, we paid attention to the *N,S*-diaryl substitution pattern of NLRP3 inflammasome inhibitors.

RESULTS AND DISCUSSION

A series of 12 substituted sulfonylureas (Table 1) was conceptualized in order to stepwise modify the structure of

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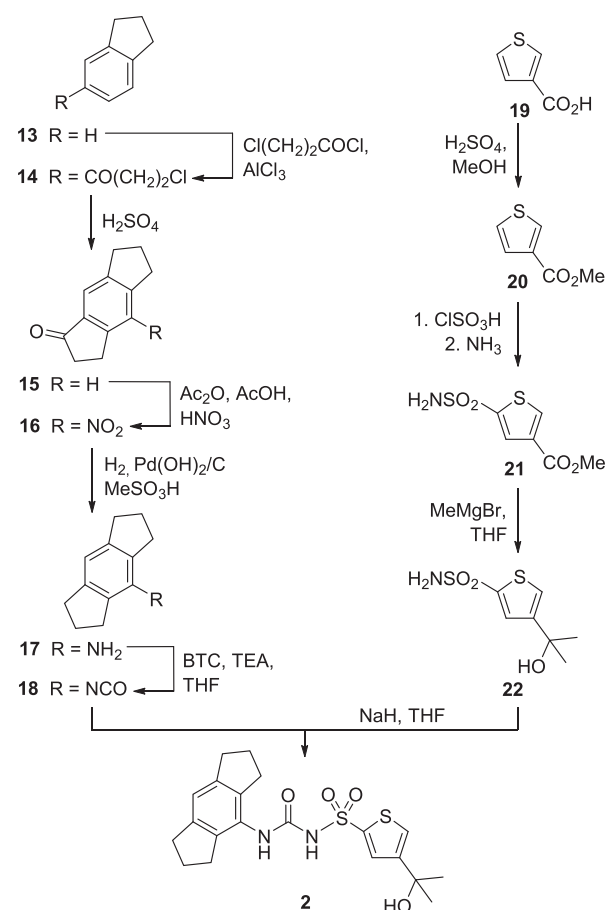
Table 1. Sulfonylureas 1–12 and Their Chemical Stability

compd		ratio of compound concentration after 8 h (%) ^a half-life (h) ^b	
		pH = 1.0	pH = 7.4
1		ratio = 50% <i>t</i> _{1/2} = 7.8 h	ratio = 99% <i>t</i> _{1/2} = n.d. ^c
2		ratio = 56% <i>t</i> _{1/2} = 8.9 h	ratio = 102% <i>t</i> _{1/2} = n.d.
3		ratio = 24% <i>t</i> _{1/2} = 3.4 h	ratio = 101% <i>t</i> _{1/2} = n.d.
4		ratio = 7% <i>t</i> _{1/2} = 1.3 h	ratio = 104% <i>t</i> _{1/2} = n.d.
5		ratio = 8% <i>t</i> _{1/2} = 2.3 h	ratio = 98% <i>t</i> _{1/2} = n.d.
6		ratio = 14% <i>t</i> _{1/2} = 2.3 h	ratio = 101% <i>t</i> _{1/2} = n.d.
7		ratio = 16% <i>t</i> _{1/2} = 2.6 h	ratio = 99% <i>t</i> _{1/2} = n.d.
8		ratio = 69% <i>t</i> _{1/2} = 15.4 h	ratio = 101% <i>t</i> _{1/2} = n.d.
9		ratio = 66% <i>t</i> _{1/2} = 13.3 h	ratio = 103% <i>t</i> _{1/2} = n.d.
10		ratio = 34% <i>t</i> _{1/2} = 5.1 h	ratio = 101% <i>t</i> _{1/2} = n.d.
11		ratio = 26% <i>t</i> _{1/2} = 4.2 h	ratio = 101% <i>t</i> _{1/2} = n.d.
12		ratio = 101% <i>t</i> _{1/2} = n.d.	ratio = 100% <i>t</i> _{1/2} = n.d.

^aAreas under the curve (AUCs) of the chromatograms after 8 h of incubation at the indicated pH were determined for the test compound and the internal standard. Ratios were calculated as a measure of stability using eq S1 (Supporting Information). ^bRatio values at nine different time points were plotted versus incubation time and fitted to the equation of exponential decay. Half-lives, *t*_{1/2}, were calculated using eq S2 (Supporting Information). ^cNot detectable; no decomposition observed within 8 h.

MCC950 (1). The preparation of one representative, compound 2, is depicted in Scheme 1, whereas all other

Scheme 1. Synthesis of Sulfonylurea 2



syntheses are provided in the Supporting Information. There is extensive patent literature on MCC950 and its relatives. Some of our test compounds appear in patents,^{16,17} which is specified in the Supporting Information. We conceived a common strategy toward these sulfonylureas. In the case of product 2, one branch of the convergent synthesis led to 1,2,3,5,6,7-hexahydro-*s*-indacen-4-amine (17),⁴ a key building block for particularly active NLRP3 inhibitors. Indane (13) was subjected to a Friedel–Crafts acylation with 3-chloropropionyl chloride in dichloromethane in the presence of aluminum chloride. The resulting ketone 14 underwent Friedel–Crafts alkylation under much harsher conditions with concd H₂SO₄ to afford the tricyclic intermediate 15. Deviating from the reported procedure,⁴ the substrate for the subsequent nitration was dissolved in a mixture of CH₂Cl₂, acetic anhydride, and acetic acid,¹⁸ and fuming HNO₃ was slowly added at 0 °C. Both functionalities of the resulting nitro ketone 16 were reduced with Pearlman's catalyst with additive methanesulfonic acid. We obtained the hexahydro-*s*-indacen-4-amine 17 after column chromatography as colorless needles.

The second branch toward sulfonylurea 2 started with thiophene-3-carboxylic acid (19), which was esterified to 20. The following one-pot synthesis involved chlorosulfonylation and ammonolysis to produce the thiophene sulfonamide 21. In order to introduce the desired tertiary alcohol group, a double Grignard reaction with methyl magnesium bromide was employed for the synthesis of 22. Next, we performed the convergent step to the final sulfonylurea 2. For this purpose, triethylamine (TEA) was added to a suspension of bis-

(trichloromethyl) carbonate (BTC) in THF at 0 °C, and a solution of the hexahydro-*s*-indacene-4-amine **17** in THF was added slowly to avoid the formation of a symmetrical urea. The isocyanate **18** was obtained after separation of insoluble material and, without further purification, combined with a mixture of sulfonamide **22** and sodium hydride in THF to afford product **2**.¹⁶

The first branch to **17** and **18** was also utilized for sulfonylureas **1**,⁴ **3**,¹⁷ **4**,¹⁷ **5**, and **6**¹⁹ (Table 1), who share the hexahydro-*s*-indacene substituent with **2**. The analogous route via Friedel–Crafts chemistry to the octahydroanthracene building block for the preparation of **7** was less advantageous than the catalytic hydrogenation of 9-aminoanthracene (see the Supporting Information, Scheme S1).

The furan analogue of compound **22** was in principal prepared as shown in the right branch of Scheme 1 starting from ethyl 3-furoate, but the conditions of the chlorosulfonylation and ammonolysis had to be precisely adapted (Scheme S3).²⁰ The final step toward all synthesized sulfonylureas was performed in an analogous manner by reacting the respective isocyanates and sulfonamides.

This series of sulfonylureas was subjected to stability assays using an high-performance liquid chromatography (HPLC) method. We analyzed five different pH conditions. Data are listed in Table 1 (see also Table S1 and Figures S1–S12). By trend, all compounds with calculated pK_a values in the range of 4–5.5 (<https://scifinder-n.cas.org>) showed a high stability at pHs 7.4 and 8.4 but underwent decomposition at pH 1 (Figure 1) and pH 4 to form the corresponding aromatic primary amine and the corresponding sulfonamide (see Figure S13).

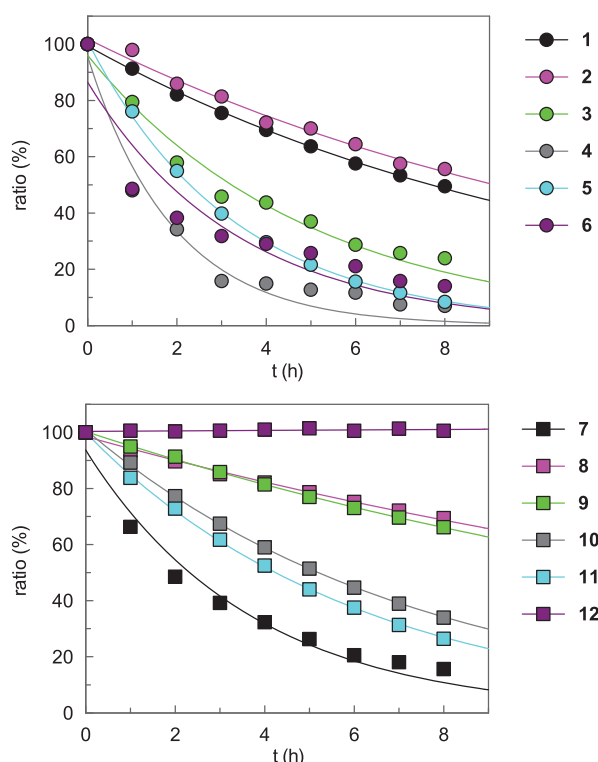


Figure 1. Time course of decomposition of sulfonylureas **1**–**12**. Compounds (0.1 mM) were incubated at 37 °C for 8 h at pH 1 (0.1 M HCl, 20% MeCN) in the presence of an internal standard (0.1 mM) and subjected to HPLC analysis. The ratios are plotted versus incubation time.

This finding clearly indicated that deprotonation at the sulfonylurea moiety stabilized the compounds. As reported for *N,S*-diaryl sulfonylurea herbicides,²¹ the neutral form was more susceptible to hydrolysis than the anionic form with the negative charge distributed throughout the sulfonylurea moiety, thus reducing the electrophilicity of the carbonyl carbon. Accordingly, liquid formulations of *N*-alkyl-*S*-aryl sulfonylureas, for example, glibenclamide, were stable,²² but not those of *N,S*-diaryl sulfonylurea herbicides. The latter could be stabilized by salt formulation.²³ Compounds **9**–**12** exhibited different stability at pH 1, reflecting the electron-withdrawing (**10**, **11**) or electron-donating (**12**) properties of the *N*-substituent. The glibenclamide fragment **12** with *N*-alkyl substitution was the only compound lacking decomposition at pH 1.

Unexpectedly, the introduction of two trimethylene bridges at the *N*-phenyl substituent decreased the stability at pH 1. The hexahydro-*s*-indacene containing compounds **4** and **6** showed 12-fold and 6-fold shorter half-lives than their benzene analogues **8** and **9**, respectively. The hexahydro-*s*-indacene derivatives **3** and **5** were unstable at pH 1 as well. To ascertain a specific effect of this tricyclic ring system, we expanded the adjacent alkylidene units and investigated the octahydroanthracene derivative **7**, which, however, was similarly unstable as its counterpart **6**. Such a degree of instability at pH 1 is not only limiting a gastrointestinal passage of compounds when applied orally but also impedes their preparation and work-up under acidic conditions.

We analyzed the influence of the tertiary alcohol group on the compound's stability and compared **1** with **3**, as well as **2** with **4**. An unforeseen extension of the half-lives by a factor of 2 and 7, respectively, was determined for **1** and **2**, caused by the presence of the 2-hydroxy-propyl-2-yl residue at position 4 of the furan or thiophene ring. Next, the affinity of selected diaryl sulfonylureas to human NLRP3 was determined by surface plasmon resonance (SPR) spectroscopy.

The recombinant biotinylated human NLRP3-NACHT domain, derived from HEK293T cells, was immobilized on a streptavidin-functionalized sensor chip, and binding of ligands was studied in the presence of ADP. Notable NLRP3 affinity was not expected for compounds **8**–**12**.²⁴ Among the sulfonylureas **1**–**7**, we selected MCC950 (**1**), **2**, **6**, and **7** for SPR analysis (Figure 2). The SPR data revealed compounds **1** and **2** to be NLRP3 ligands with high affinity (Table 2). Our finding showed that the exchange of the ring oxygen (in **1**) by sulfur (in **2**) was well tolerated, which offers justification for the development of thiophene-based NLRP3 ligands. This is also reflected in the patent literature.¹⁶ An unsubstituted aromatic *S*-substituent reduced the affinity as exemplified with compound **6**, indicating that the tertiary alcohol group (in **1** and **2**) contributed to the NLRP3–ligand interaction. Noteworthy, the replacement of the hexahydro-*s*-indacene moiety with an octahydroanthracene moiety caused the total loss of binding ability of **7**.

As a conclusion, our series of MCC950 analogues revealed that subtle changes in the inhibitor structure influenced chemical stability under acidic conditions. Truncation of the alkylidene bridges at the tricyclic unit caused an unanticipated increase of stability. However, the hexahydro-*s*-indacene moiety has commonly been established to achieve NLRP3 inhibition. Our study demonstrated that the stability of hexahydro-*s*-indacene-containing compounds can be regained through the introduction of the tertiary alcohol group, which

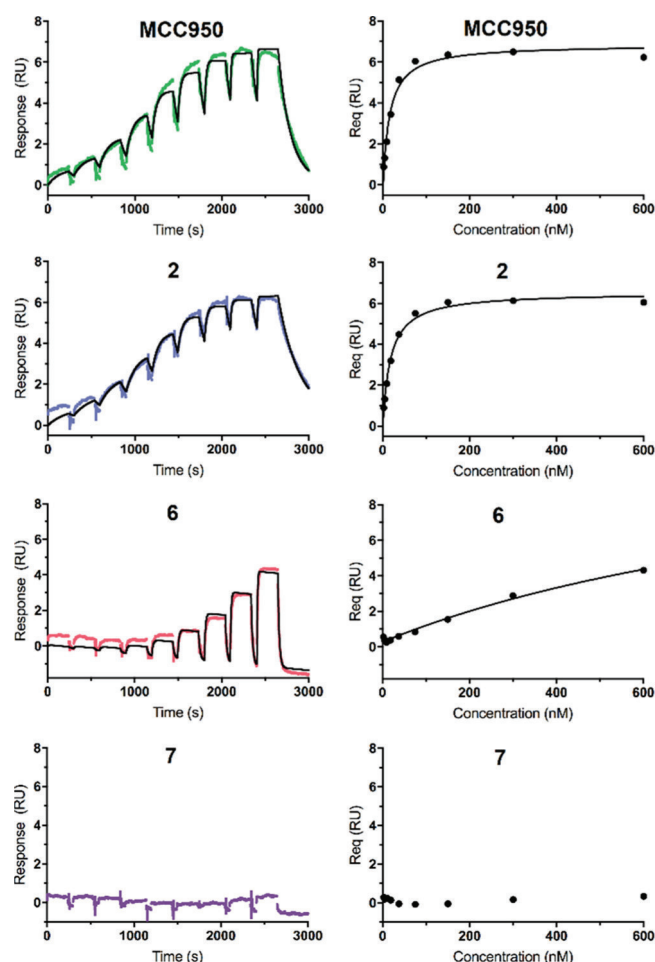


Figure 2. SPR sensorgrams (left) and saturation curves (right) of MCC950, 2, 6, and 7 injected on single channels. The chip surface was loaded with human NLRP3-NACHT. RU is the response unit, R_{eq} the steady-state response. The binding parameters are listed in Table 2. Data were fitted to a 1:1 binding model.

Table 2. SPR Binding Behavior Data of Selected Sulfonylureas to Human NLRP3^a

cmpd	k_a ($10^5 M^{-1} s^{-1}$)	k_d ($10^{-3} s^{-1}$)	$K_{D,kin}$ (nM)	$K_{D,eq}$ (nM)
1 ^b	5.3 ± 2.5	6.2 ± 1.3	15 ± 4	21 ± 4
2	2.5 ± 0.2	3.9 ± 1.1	15 ± 4	19 ± 2
6	>2.0	>25	n.d. ^c	>600 ^d
7	n.d. ^e	n.d. ^e	n.d. ^e	n.d. ^e

^aSecond-order on-rate constants for association (k_a), first-order off-rate constants for dissociation (k_d), kinetic dissociation constants ($K_{D,kin}$), and equilibrium dissociation constant ($K_{D,eq}$) for selected sulfonylureas ($n = 3$). ^bA K_D value of 10.6 nM obtained by SPR for binding of 1 to NLRP3 Δ LRR in the presence of ATP was reported.⁷

^cNot determined due to the fast binding kinetics of compound 6.

^dInsufficient saturation of immobilized NLRP3 with compound 6.

^eBinding of 7 to NLRP3 was not detectable.

additionally improved the compounds' potency. The combination of both structural features accounts for the high suitability of MCC950 as a pharmacological tool compound. With the diaryl sulfonylurea 2, we introduce a thiophene bioisostere of MCC950 (1), which was equipotent and similarly stable. This discovery suggests further synthetic options toward NLRP3 ligands based on thiophene chemistry.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsomega.2c00125>.

General information, preparation of compounds, analytical data, HPLC stability, and SPR spectroscopy measurements (PDF)

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Notes

The authors declare no competing financial interest.

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■ DEDICATION

This work is dedicated to Professor Dr. Kurt Eger on the occasion of his 80th birthday.

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Supporting Information

Structure-Stability Relationship of NLRP3 Inflammasome-Inhibiting Sulfonylureas

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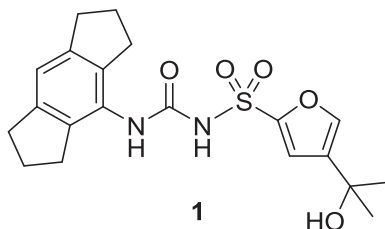
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General Information

Chemicals were purchased from ABCR, Acros Organics, BLDpharm, Carl Roth, Enamine, Fisher Scientific, Sigma Aldrich, Tokyo Chemical Industry and VWR Chemicals. Indane (**13**), thiophene-3-carboxylic acid (**19**) and, 9-nitroanthracene (**23**), 3,4-dichloroaniline (**27**), and 3-ethyl furoate (**29**) were commercially available. 4-Isocyanato-1,2,3,5,6,7-hexahydro-*s*-indacene (**18**), 9-isocyanato-1,2,3,4,5,6,7,8-octahydroanthracene (**26**), and 1,2-dichloro-4-isocyanatobenzene (**28**) were prepared as noted below and used in the next steps without further purification.

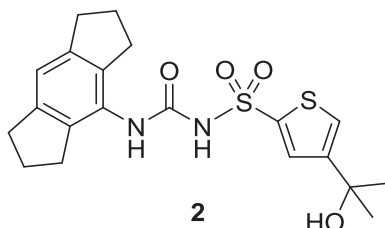
Thin layer chromatography was carried out with pre-coated silica gel (60 F254) aluminum sheets from Merck. Detection was performed with UV light at 254 and 360 nm or with AgNO₃ staining. Acros Organics silica gel 60 (70–230 mesh) was taken for preparative column chromatography. Preparative silica gel flash column chromatography was performed on an Interchim Puriflash PF420 system with diode-array detection (DAD) from 200 to 400 nm. Melting points were measured on a Büchi 510 oil bath apparatus. HR-ESI-MS spectra were recorded on a Bruker micrOTOF-Q mass spectrometer coupled with a HPLC Dionex UltiMate 3000 or a LTQ Orbitrap XL. ESI-MS mass spectra were recorded on an API2000 mass spectrometer coupled with an Agilent HPLC HP1100 using an EC50/2 Nucleodur C18 Gravity 3 μ m column or on a Agilent Infinity Lab LC/MSD-system coupled with a Agilent HPLC 1260 Infinity II using a EC50/2 Nucleodur C18 Gravity 3 μ m column. The purity of synthesized compounds was determined by HPLC-DAD. NMR spectra were recorded on a Bruker Avance DRX 500 (500 MHz ¹H NMR, 126 MHz ¹³C NMR) and a Bruker Avance III 600 (600 MHz ¹H NMR, 151 MHz ¹³C NMR). Chemical shifts are given in parts per million (ppm) referring to the signal center using the solvent peaks for reference: DMSO-*d*₆ (2.49/39.7).

Preparation of Compounds



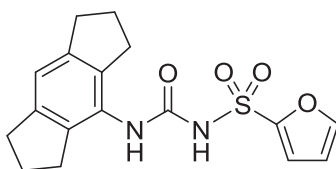
***N*-((1,2,3,5,6,7-Hexahydro-*s*-indacen-4-yl)carbamoyl)-4-(2-hydroxypropan-2-yl)furan-2-sulfonamide (**1**).** Under nitrogen, triphosgene (BTC, 267 mg, 0.9 mmol) was dissolved in dry THF (20 mL). The mixture was stirred and cooled to 0 °C for 30 min. TEA (182 mg, 1.8 mmol) was added. 1,2,3,5,6,7-Hexahydro-*s*-indacen-4-amine (**17**, 156 mg, 0.9 mmol) was dissolved in dry THF (20 mL) and dropwise added over 30 min. The mixture was stirred for additional 30 min at room temperature and then 30 min at 75 °C. The solvent was evaporated and the residue was taken up in dry THF (20 mL), filtered and concentrated to yield 4-isocyanato-1,2,3,5,6,7-hexahydro-*s*-indacene (**18**). 4-(2-Hydroxypropan-2-yl)furan-2-sulfonamide (**31**, 185 mg, 0.9 mmol) was dissolved in dry THF (10 mL). NaH (60% dispersion in mineral oil, 65 mg, 1.6 mmol) was added and the reaction mixture was stirred at 0 °C under nitrogen for 30 min. The crude 4-isocyanato-1,2,3,5,6,7-hexahydro-*s*-indacene (**18**) was dissolved in dry THF (5 mL) and added in one portion. The reaction mixture was stirred for 2 h at room temperature under nitrogen. The organic solvent was removed under reduced pressure. The residue was dispersed in H₂O (15 mL) and acidified with 2N HCl. The precipitate was collected, washed with H₂O (3 $\times$ 5 mL), dried, and washed with hexane (3 $\times$ 5 mL). It was dried under high vacuum to yield **1** as a white solid (336 mg).

Yield 92%; mp: >140 °C (decomposition), lit.¹ mp: 239 °C (sodium salt); ¹H NMR (600 MHz, DMSO-*d*₆) δ 1.39 (s, 6H), 1.94 (quint, *J* = 7.4 Hz, 4H), 2.59 (t, *J* = 7.3 Hz, 4H), 2.79 (t, *J* = 7.4 Hz, 4H), 5.11 (s, 1H), 6.95 (s, 1H), 7.26 (s, 1H), 7.82 (s, 1H), 8.10 (s, 1H), 10.97 (s, 1H); ¹³C NMR (151 MHz, DMSO-*d*₆) δ 25.00, 30.05, 30.92, 32.41, 66.55, 116.50, 118.12, 128.43, 137.14, 137.30, 141.97, 143.10, 147.22, 148.36; **LC-MS(ESI)** (90% H₂O + 2 mM NH₄Ac to 100% MeCN in 10 min, then 100% MeCN to 15 min, DAD 200-600 nm), *t*_R = 4.08 min, 96% purity, *m/z* [M+H]⁺ calcd for C₂₀H₂₄N₂O₅S 405.1, found 405.2; **HRMS (ESI)** *m/z* [M+Na]⁺ calcd for C₂₀H₂₄N₂O₅S 427.1298, found 427.1310.



***N*-((1,2,3,5,6,7-Hexahydro-*s*-indacen-4-yl)carbamoyl)-4-(2-hydroxypropan-2-yl)thiophene-2-sulfonamide (2).** Under nitrogen, triphosgene (BTC, 534 mg, 1.8 mmol) was dissolved in dry THF (30 mL). The mixture was stirred and cooled to 0 °C for 30 min. TEA (364 mg, 3.6 mmol) was added. 1,2,3,5,6,7-Hexahydro-*s*-indacen-4-amine (**17**, 312 mg, 1.8 mmol) was dissolved in dry THF (30 mL) and dropwise added over 30 min. The mixture was stirred for additional 30 min at room temperature and then 30 min at 75 °C. The solvent was evaporated and the residue was taken up in dry THF (30 mL), filtered and concentrated to yield 4-isocyanato-1,2,3,5,6,7-hexahydro-*s*-indacene (**18**). 4-(2-Hydroxypropan-2-yl)thiophene-2-sulfonamide (**22**, 376 mg, 1.7 mmol) was dissolved in dry THF (15 mL). NaH (60 % dispersion in mineral oil, 130 mg, 3.24 mmol) was added and the reaction mixture was stirred at 0 °C under nitrogen for 30 min. The crude 4-isocyanato-1,2,3,5,6,7-hexahydro-*s*-indacene (**18**) was dissolved in dry THF (7.5 mL) and added in one portion. The reaction mixture was stirred for 2 h at room temperature under nitrogen atmosphere. The organic solvent was removed under reduced pressure. The residue was dispersed in H₂O (30 mL) and acidified with 2 N HCl. The precipitate was collected and dried under high vacuum. The crude product was purified by silica column chromatography using petroleum ether/EtOAc (1+1) as eluent to yield **2** as a grey solid (492 mg).

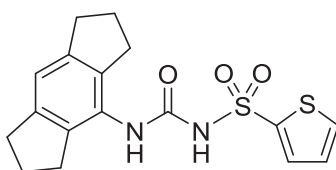
Yield 69%; mp: >130 °C (decomposition), lit.² mp: 133-134 °C; ¹H NMR (500 MHz, DMSO-*d*₆) δ 1.42 (s, 6H), 1.94 (quint, *J* = 7.3 Hz, 4H), 2.59 (t, *J* = 7.4 Hz, 4H), 2.79 (t, *J* = 7.4 Hz, 4H), 5.23 (s, 1H), 6.94 (s, 1H), 7.69 – 7.71 (m, 1H), 7.76 – 7.77 (m, 1H), 8.10 (s, 1H), 10.60 (s, 1H); ¹³C NMR (126 MHz, DMSO-*d*₆) δ 24.98, 30.06, 31.32, 32.40, 69.32, 118.02, 126.46, 128.55, 131.85, 137.21, 139.85, 143.07, 148.85, 152.47; **LC-MS (ESI)** (90% H₂O + 2 mM NH₄Ac to 100% MeOH + 2 mM NH₄Ac in 10 min, then 100% MeOH + 2 mM NH₄Ac to 20 min, DAD 220-400 nm), *t*_R = 9.85 min, 97% purity, *m/z* [M+H]⁺ calcd for C₂₀H₂₄N₂O₄S₂ 421.1, found 421.4; **HRMS (ESI)** *m/z* [M+Na]⁺ calcd for C₂₀H₂₄N₂O₄S₂ 443.1070, found 443.1092.



3

***N*-((1,2,3,5,6,7-Hexahydro-*s*-indacen-4-yl)carbamoyl)furan-2-sulfonamide (3).** Under nitrogen, triphosgene (BTC, 202 mg, 0.68 mmol) was dissolved in dry THF (15 mL). The mixture was stirred and cooled to 0 °C for 30 min. TEA (138 mg, 1.36 mmol) was added. 1,2,3,5,6,7-Hexahydro-*s*-indacen-4-amine (**17**, 118 mg, 0.68 mmol) was dissolved in dry THF (15 mL) and dropwise added over 30 min. The mixture was stirred for additional 30 min at room temperature and then 30 min at 75 °C. The solvent was evaporated and the residue was taken up in dry THF (10 mL), filtered and concentrated to yield 4-isocyanato-1,2,3,5,6,7-hexahydro-*s*-indacene (**18**). Furan-2-sulfonamide (111 mg, 0.68 mmol) was dissolved in dry THF (15 mL). NaH (60% dispersion in mineral oil, 49 mg, 1.22 mmol) was added and the reaction mixture was stirred at 0 °C under nitrogen for 30 min. The crude 4-isocyanato-1,2,3,5,6,7-hexahydro-*s*-indacene (**18**) was dissolved in dry THF (3.5 mL) and added in one portion. The reaction mixture was stirred for 2 h at room temperature under nitrogen atmosphere. The organic solvent was removed under reduced pressure. The residue was dispersed in H₂O (15 mL) and acidified with 2 N HCl. The built precipitate was collected, washed with H₂O and EtOAc and was dried under high vacuum to yield **3** as a white solid (204 mg).

Yield 87%; mp: 210-212 °C; ¹H NMR (500 MHz, DMSO-*d*₆) δ 1.95 (quint, *J* = 7.4 Hz, 4H), 2.59 (t, *J* = 7.4 Hz, 4H), 2.79 (t, *J* = 7.3 Hz, 4H), 6.72 (dd, *J* = 3.2, 1.9 Hz, 1H), 6.95 (s, 1H), 7.26 (d, *J* = 3.7 Hz, 1H), 8.03 (s, 1H), 8.11 (s, 1H), 10.91 (s, 1H); ¹³C NMR (126 MHz, DMSO-*d*₆) δ 24.98, 30.00, 32.39, 111.70, 117.77, 118.09, 128.41, 137.25, 143.08, 147.47, 147.50, 148.35; **LC-MS(ESI)** (90% H₂O + 2 mM NH₄Ac to 100% MeOH + 2 mM NH₄Ac in 10 min, then 100% MeOH + 2 mM NH₄Ac to 20 min, DAD 220-400 nm), *t*_R = 9.57 min, 96% purity, *m/z* [M+H]⁺ calcd for C₁₇H₁₈N₂O₄S 347.1, found 347.2; **HRMS (ESI)** *m/z* [M+H]⁺ calcd for C₁₇H₁₈N₂O₄S 347.1060, found 347.1075, lit.³ *m/z* [M-H]⁻ calcd 345.0987, found 345.0866.

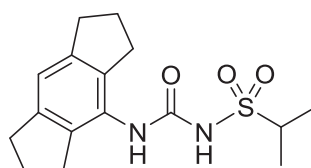


4

***N*-((1,2,3,5,6,7-Hexahydro-*s*-indacen-4-yl)carbamoyl)thiophene-2-sulfonamide (4).** Under nitrogen, triphosgene (BTC, 534 mg, 1.8 mmol) was dissolved in dry THF (30 mL). The mixture was stirred and cooled to 0 °C for 30 min. TEA (364 mg, 3.6 mmol) was added. 1,2,3,5,6,7-Hexahydro-*s*-indacen-4-amine (**17**, 312 mg, 1.8 mmol) was dissolved in dry THF (30 mL) and dropwise added over 30 min. The mixture was stirred for additional 30 min at room temperature and then 30 min at 75 °C. The solvent was evaporated and the residue was taken up in dry THF (30 mL), filtered and concentrated to yield 4-isocyanato-1,2,3,5,6,7-hexahydro-*s*-indacene (**18**). Thiophene-2-sulfonamide (294 mg, 1.8 mmol) was dissolved in dry THF (15 mL). NaH (60% dispersion in mineral oil, 130 mg, 3.24 mmol) was added and the reaction mixture was stirred at 0 °C under nitrogen for 30 min. The crude 4-isocyanato-1,2,3,5,6,7-hexahydro-*s*-indacene (**18**) was dissolved in dry THF (7.5 mL) and added in one portion. The reaction mixture was stirred for 4 h at room temperature under nitrogen. The organic solvent was removed under reduced pressure. The residue was dispersed in H₂O (30 mL) and

acidified with 2 N HCl. The precipitate was collected, washed with H₂O (3 × 5 mL) and dried under high vacuum to yield **4** as a white solid (542 mg).

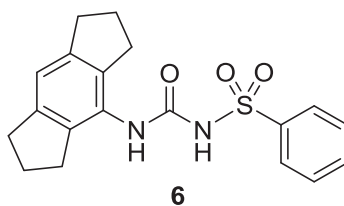
Yield 83%; mp: >230 °C (decomposition); ¹H NMR (600 MHz, DMSO-*d*₆) δ 1.94 (quint, *J* = 7.5 Hz, 4H), 2.58 (t, *J* = 7.4 Hz, 4H), 2.79 (t, *J* = 7.4 Hz, 4H), 6.94 (s, 1H), 7.19 (dd, *J* = 5.0, 3.8 Hz, 1H), 7.77 (dd, *J* = 3.7, 1.4 Hz, 1H), 8.01 (dd, *J* = 5.0, 1.4 Hz, 1H), 8.11 (s, 1H), 10.88 (s, 1H); ¹³C NMR (151 MHz, DMSO-*d*₆) δ 25.00, 30.05, 32.41, 118.05, 127.27, 128.52, 133.48, 133.98, 137.21, 140.39, 143.09, 148.87; LC-MS (ESI) (90% H₂O + 2 mM NH₄Ac to 100% MeOH + 2 mM NH₄Ac in 10 min, then 100% MeOH + 2 mM NH₄Ac to 20 min, DAD 220-400 nm), *t*_R = 9.95 min, 97% purity, *m/z* [M+H]⁺ calcd for C₁₇H₁₈N₂O₃S₂ 363.1, found 363.2; HRMS (ESI) *m/z* [M+Na]⁺ calcd for C₁₇H₁₈N₂O₃S₂ 385.0658, found 385.0651, lit.³ *m/z* [M+H]⁺ calcd 363.0832, found 363.0819.



5

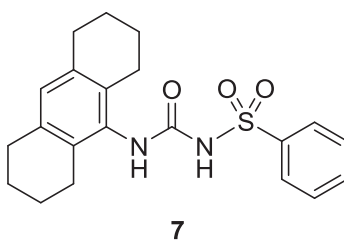
N-((1,2,3,5,6,7-Hexahydro-s-indacen-4-yl)carbamoyl)propane-2-sulfonamide (5). Under nitrogen, triphosgene (BTC, 202 mg, 0.68 mmol) was dissolved in dry THF (15 mL). The mixture was stirred and cooled to 0 °C for 30 min. TEA (138 mg, 1.36 mmol) was added. 1,2,3,5,6,7-Hexahydro-s-indacen-4-amine (**17**, 118 mg, 0.68 mmol) was dissolved in dry THF (15 mL) and dropwise added over 30 min. The mixture was stirred for additional 30 min at room temperature and then 30 min at 75 °C. The solvent was evaporated and the residue was taken up in dry THF (10 mL), filtered and concentrated to yield 4-isocyanato-1,2,3,5,6,7-hexahydro-s-indacene (**18**). Propane-2-sulfonamide (84 mg, 0.68 mmol) was dissolved in dry THF (10 mL). NaH (60 % dispersion in mineral oil, 49 mg, 1.22 mmol) was added and the reaction mixture was stirred at 0 °C under nitrogen for 30 min. The crude 4-isocyanato-1,2,3,5,6,7-hexahydro-s-indacene (**18**) was dissolved in dry THF (3.5 mL) and added in one portion. The reaction mixture was stirred for 2 h at room temperature under nitrogen atmosphere. The organic solvent was removed under reduced pressure. The residue was dispersed in H₂O (15 mL) and acidified with 2 N HCl. The precipitate was collected, washed with H₂O (3 × 5 mL), dried, and washed with EtOAc (3 × 5 mL). It was dried under high vacuum to yield **5** as a white solid (216 mg).

Yield 99%; mp: 178-180 °C; ¹H NMR (600 MHz, DMSO-*d*₆) δ 1.31 (d, *J* = 6.9 Hz, 6H), 1.99 (quint, *J* = 7.6 Hz, 4H), 2.70 (t, *J* = 7.2 Hz, 4H), 2.81 (t, *J* = 7.3 Hz, 4H), 3.70 (quint, *J* = 7.0 Hz, 1H), 6.96 (s, 1H), 8.16 (s, 1H), 10.21 (s, 1H); ¹³C NMR (151 MHz, DMSO-*d*₆) δ 15.67, 25.04, 30.16, 32.43, 52.57, 117.99, 128.66, 137.15, 143.11, 149.63; LC-MS (ESI) (90% H₂O + 2 mM NH₄Ac to 100% MeOH + 2 mM NH₄Ac in 10 min, then 100% MeOH + 2 mM NH₄Ac to 20 min, DAD 210-400 nm), *t*_R = 10.76 min, 93% purity, *m/z* [M+H]⁺ calcd for C₁₆H₂₂N₂O₃S 323.1, found 323.1; HRMS (ESI) *m/z* [M+H]⁺ calcd for C₁₆H₂₂N₂O₃S 323.1424, found 323.1433.



***N*-((1,2,3,5,6,7-Hexahydro-*s*-indacen-4-yl)carbamoyl)benzenesulfonamide (6).** Under nitrogen, triphosgene (BTC, 534 mg, 1.8 mmol) was dissolved in dry THF (30 mL). The mixture was stirred and cooled to 0 °C for 30 min. TEA (364 mg, 3.6 mmol) was added. 1,2,3,5,6,7-Hexahydro-*s*-indacen-4-amine (**17**, 312 mg, 1.8 mmol) was dissolved in dry THF (30 mL) and dropwise added over 30 min. The mixture was stirred for additional 30 min at room temperature and then 30 min at 75 °C. The solvent was evaporated and the residue was taken up in dry THF (30 mL), filtered and concentrated to yield 4-isocyanato-1,2,3,5,6,7-hexahydro-*s*-indacene (**18**). Benzenesulfonamide (252 mg, 1.6 mmol) was dissolved in dry THF (15 mL). NaH (60% dispersion in mineral oil, 115 mg, 2.9 mmol) was added and the reaction mixture was stirred at 0 °C under nitrogen for 30 min. The crude 4-isocyanato-1,2,3,5,6,7-hexahydro-*s*-indacene (**18**) was dissolved in dry THF (7.5 mL) and added in one portion. The reaction mixture was stirred for 4 h at room temperature under nitrogen. The organic solvent was removed under reduced pressure. The residue was partitioned between EtOAc (50 mL) and saturated NH₄Cl solution (50 mL). The organic layer was separated. The aqueous phase was extracted with EtOAc (2 × 50 mL). The combined organic layers were dried over Na₂SO₄, filtered and concentrated. The crude product was purified by silica gel column chromatography using petroleum ether/EtOAc (1+1) as eluent to yield **6** as a white solid (334 mg).

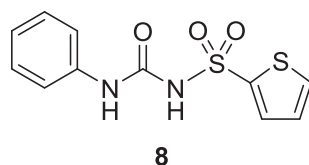
Yield 59%; mp: >230°C (decomposition); ¹H NMR (500 MHz, DMSO-*d*₆) δ 1.91 (quint, *J* = 7.4 Hz, 4H), 2.53 (t, *J* = 7.4 Hz, 4H), 2.77 (t, *J* = 7.4 Hz, 4H), 6.92 (s, 1H), 7.59 – 7.72 (m, 3H), 7.95 (d, *J* = 7.8 Hz, 2H), 8.09 (s, 1H), 10.69 (s, 1H); ¹³C NMR (126 MHz, DMSO-*d*₆) δ 24.95, 29.99, 32.38, 117.97, 127.15, 128.52, 128.95, 133.18, 137.16, 140.01, 143.04, 148.94; LC-MS(ESI) (90% H₂O + 2 mM NH₄Ac to 100% MeCN in 10 min, then 100% MeCN to 20 min, DAD 220–400 nm), *t*_R = 8.58 min, 98% purity, *m/z* [M+H]⁺ calcd for C₁₉H₂₀N₂O₃S 357.1, found 357.2; HRMS (ESI) *m/z* [M+Na]⁺ calcd for C₁₉H₂₀N₂O₃S 379.1087, found 379.1103, lit.⁴ MS (ESI) *m/z* [M+H]⁺ calcd 357.13, found 357.05.



***N*-((1,2,3,4,5,6,7,8-Octahydroanthracen-9-yl)carbamoyl)benzenesulfonamide (7).** Under nitrogen, triphosgene (BTC, 267 mg, 0.9 mmol) was dissolved in dry THF (20 mL). The mixture was stirred and cooled to 0 °C for 30 min. TEA (182 mg, 1.8 mmol) was added. 1,2,3,4,5,6,7,8-Octahydroanthracen-9-amine (**25**, 181 mg, 0.9 mmol) was dissolved in dry THF (20 mL) and dropwise added over 30 min. The mixture was stirred for additional 30 min at room temperature and then 30 min at 75 °C. The solvent was evaporated and the residue was taken up in dry THF (20 mL), filtered and concentrated to yield 9-isocyanato-1,2,3,4,5,6,7,8-octahydroanthracene (**26**). Benzenesulfonamide (142 mg, 0.9 mmol) was dissolved in dry THF (10 mL). NaH (60% dispersion in

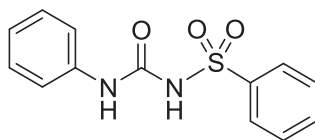
mineral oil, 65 mg, 1.6 mmol) was added and the reaction mixture was stirred at 0 °C under nitrogen for 30 min. The crude 9-isocyanato-1,2,3,4,5,6,7,8-octahydroanthracene (**26**) was dissolved in dry THF (5 mL) and added in one portion. The reaction mixture was stirred for 2 h at room temperature under nitrogen. The organic solvent was removed under reduced pressure. The residue was dispersed in H₂O (15 mL) and acidified with 2 N HCl. The precipitate was collected, washed with H₂O (3 × 5 mL), dried, and washed with hexane (3 × 5 mL). It was dried under high vacuum to yield **7** as a white solid (295 mg).

Yield 85%; mp: >220 °C (decomposition); ¹H NMR (500 MHz, DMSO-*d*₆) δ 1.53 – 1.62 (m, 8H), 2.09 – 2.34 (m, 4H), 2.56 – 2.61 (m, 4H), 6.69 (s, 1H), 7.58 – 7.64 (m, 2H), 7.67 – 7.73 (m, 2H), 7.90 – 7.96 (m, 2H), 10.87 (s, 1H); ¹³C NMR (126 MHz, DMSO-*d*₆) δ 22.43, 22.57, 24.24, 28.72, 127.24, 128.22, 128.98, 131.62, 132.87, 133.22, 134.06, 140.02, 149.61; LC-MS(ESI) (90% H₂O + 2 mM NH₄Ac to 100% MeCN in 10 min, then 100% MeCN to 15 min, DAD 200-600 nm), *t*_R = 5.36 min, 99% purity, *m/z* [M+H]⁺ calcd for C₂₁H₂₄N₂O₃S 385.2, found 385.2; HRMS(ESI) *m/z* [M+Na]⁺ calcd for C₂₁H₂₄N₂O₃S 407.1401, found 407.1401.



N-(Phenylcarbamoyl)thiophene-2-sulfonamide (8). Thiophene-2-sulfonamide (490 mg, 3 mmol) was dissolved in dry THF (15 mL). NaH (60 % dispersion in mineral oil, 216 mg, 5.4 mmol) was added and the reaction mixture was stirred at 0 °C under argon for 30 min. Phenyl isocyanate (357 mg, 3 mmol) was dissolved in dry THF (7.5 mL) and added in one portion. The reaction mixture was stirred for 4 h at room temperature under argon. The organic solvent was removed under reduced pressure. The residue was dispersed in H₂O (30 mL) and acidified with 2 N HCl. The precipitate was collected, washed with H₂O (3 × 5 mL), dried, and washed with CH₂Cl₂ (3 × 5 mL). It was dried under high vacuum to yield a white solid (358 mg).

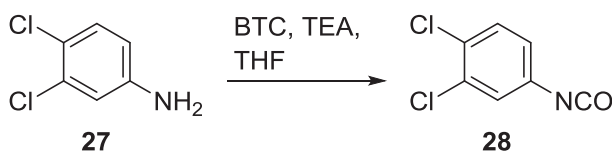
Yield 42%; mp: 160-162 °C; ¹H NMR (600 MHz, DMSO-*d*₆) δ 7.02 – 7.05 (m, 1H), 7.20 (dd, *J* = 5.0, 3.8 Hz, 1H), 7.26 – 7.29 (m, 2H), 7.36 – 7.38 (m, 2H), 7.80 (dd, *J* = 3.8, 1.5 Hz, 1H), 8.01 (dd, *J* = 5.1, 1.4 Hz, 1H), 8.84 (s, 1H), 10.94 (s, 1H), lit.⁵ ¹H NMR (400 MHz, DMSO-*d*₆) δ 7.04 (d, *J* = 7.3 Hz, 1H), 7.20 (dd, *J* = 4.9, 3.9 Hz, 1H), 7.28 (t, *J* = 7.9 Hz, 2H), 7.37 (d, *J* = 7.6 Hz, 2H), 7.81 (dd, *J* = 3.8, 1.3 Hz, 1H), 8.02 (dd, *J* = 5.0, 1.3 Hz, 1H), 8.89 (s, 1H), 10.94 (s, 1H); ¹³C NMR (151 MHz, DMSO-*d*₆) δ 119.07, 123.31, 127.40, 128.82, 133.69, 134.14, 137.92, 140.30, 149.21; LC-MS(ESI) (90% H₂O + 2 mM NH₄Ac to 100% MeOH + 2 mM NH₄Ac in 10 min, then 100% MeOH + 2 mM NH₄Ac to 20 min, DAD 220-400 nm), *t*_R = 5.21 min, 100% purity, *m/z* [M+Na]⁺ calcd for C₁₁H₁₀N₂O₃S₂ 283.0, found 283.1; HRMS(ESI) *m/z* [M+Na]⁺ calcd for C₁₁H₁₀N₂O₃S₂ 305.0036, found 305.0025.



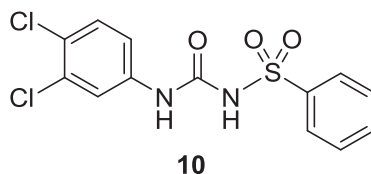
9

***N*-(Phenylcarbamoyl)benzenesulfonamide (9).** Benzenesulfonamide (472 mg, 3 mmol) was dissolved in dry THF (15 mL). NaH (60 % dispersion in mineral oil, 216 mg, 5.4 mmol) was added and the reaction mixture was stirred at 0 °C under argon for 30 min. Phenyl isocyanate (357 mg, 3 mmol) was dissolved in dry THF (7.5 mL) and added in one portion. The reaction mixture stirred for 4 h at room temperature under argon. The reaction was quenched by dropwise adding of H₂O (1 mL) and the organic solvent was removed under reduced pressure. The residue was partitioned between EtOAc (100 mL) and saturated NH₄Cl solution (100 mL). The organic layer was separated. The aqueous phase was extracted with EtOAc (2 × 50 mL). The combined organic layers were dried over Na₂SO₄, filtered and concentrated. The crude product was purified by silica gel column chromatography using petroleum ether/EtOAc (2+1) as eluent to yield **9** as a white solid (497 mg).

Yield 60%; mp: 156-158 °C, lit.⁶ mp: 158-160 °C; ¹H NMR (600 MHz, DMSO-*d*₆) δ 6.99 – 7.02 (m, 1H), 7.23 – 7.27 (m, 2H), 7.31 – 7.35 (m, 2H), 7.60 – 7.64 (m, 2H), 7.67 – 7.71 (m, 1H), 7.96 – 7.98 (m, 2H), 8.81 (s, 1H), 10.72 (s, 1H); ¹³C NMR (151 MHz, DMSO-*d*₆) δ 118.93, 123.14, 127.35, 128.77, 128.99, 133.21, 138.04, 140.12, 149.45; **LC-MS(ESI)** (90% H₂O + 2 mM NH₄Ac to 100% MeOH + 2 mM NH₄Ac in 10 min, then 100% MeOH + 2 mM NH₄Ac to 20 min, DAD 220-400 nm), *t*_R = 6.38 min, 100% purity, *m/z* [M+H]⁺ calcd for C₁₃H₁₂N₂O₃S 277.1, found 277.2; **HRMS(ESI)** *m/z* [M+Na]⁺ calcd for C₁₃H₁₂N₂O₃S 299.0461, found 299.0476.

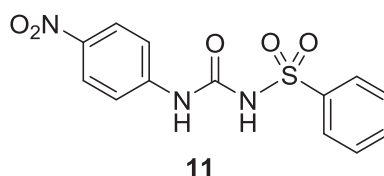


Scheme S1. Synthesis of 3,4-dichloroisocyanate 28



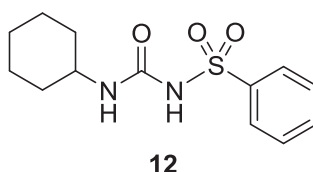
N-((3,4-Dichlorophenyl)carbamoyl)benzenesulfonamide (10). Under argon, triphosgene (BTC, 534 mg, 1.8 mmol) was dissolved in dry THF (30 mL). The mixture was stirred and cooled to 0 °C for 30 min. TEA (364 mg, 3.6 mmol) was added. 3,4-Dichloroaniline (**27**, 297 mg, 1.8 mmol) was dissolved in dry THF (30 mL) and dropwise added over 30 min. The mixture was stirred for additional 30 min at room temperature and then 30 min at 75 °C. The solvent was evaporated and the residue was taken up in dry THF (30 mL), filtered and the filtrate was concentrated to yield 1,2-dichloro-4-isocyanatobenzene (**28**). Benzenesulfonamide (283 mg, 1.8 mmol) was dissolved in dry THF (15 mL). NaH (60% dispersion in mineral oil, 130 mg, 3.24 mmol) was added and the reaction mixture was stirred at 0 °C under argon for 30 min. The crude 1,2-dichloro-4-isocyanatobenzene (**28**) was dissolved in dry THF (7.5 mL) and added in one portion. The reaction mixture was stirred for 4 h at room temperature under argon. The reaction was quenched by dropwise adding of H₂O (1 mL) and the organic solvent was removed under reduced pressure. The residue was partitioned between EtOAc (100 mL) and saturated NH₄Cl solution (100 mL). The organic layer was separated. The aqueous phase was extracted with EtOAc (2 × 50 mL). The combined organic layers were dried over Na₂SO₄, filtered and concentrated. The crude product was purified by silica column chromatography using petroleum ether/EtOAc (1+1) as eluent to yield a white solid (216 mg).

Yield 35%; mp: 168-170 °C, lit.⁶ mp: 194-195 °C; ¹H NMR (600 MHz, DMSO-*d*₆) δ 7.24 – 7.33 (m, 1H), 7.43 – 7.54 (m, 1H), 7.59 – 7.64 (m, 2H), 7.65 – 7.73 (m, 2H), 7.90 – 8.00 (m, 2H), 9.14 (s, 1H), 11.10 (s, 1H); ¹³C NMR (151 MHz, DMSO-*d*₆) δ 118.97, 119.93, 124.23, 127.31, 128.91, 130.54, 130.95, 133.00, 138.73, 140.51, 150.31; **LC-MS(ESI)** (90% H₂O + 2 mM NH₄Ac to 100% MeOH + 2 mM NH₄Ac in 10 min, then 100% MeOH + 2 mM NH₄Ac to 20 min, DAD 220-400 nm), *t*_R = 9.31 min, 100% purity, *m/z* [M+H]⁺ calcd for C₁₃H₁₀Cl₂N₂O₃S 345.0, found 345.1; **HRMS(ESI)** *m/z* [M+Na]⁺ calcd for C₁₃H₁₀Cl₂N₂O₃S 388.9501, found 388.9511.



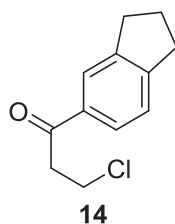
N-((4-Nitrophenyl)carbamoyl)benzenesulfonamide (11). Benzenesulfonamide (472 mg, 3 mmol) was dissolved in dry THF (15 mL). NaH (60 % dispersion in mineral oil, 216 mg, 5.4 mmol) was added and the reaction mixture was stirred at 0 °C under argon for 30 min. 4-Nitrophenyl isocyanate (492 mg, 3 mmol) was dissolved in dry THF (7.5 mL) and added in one portion. The reaction mixture stirred for 4 h at room temperature under argon. The organic solvent was removed under reduced pressure. The residue was dispersed in H₂O (30 mL) and acidified with 2 N HCl. The precipitate was collected, washed with H₂O (3 × 5 mL) and was dried under high vacuum to yield **11** as a yellow solid (713 mg).

Yield 74%; mp: >230 °C (decomposition), lit.⁷ mp: 244-245 °C; ¹H NMR (600 MHz, DMSO-*d*₆) δ 7.58 – 7.60 (m, 2H), 7.63 – 7.66 (m, 2H), 7.70 – 7.73 (m, 1H), 7.98 – 8.00 (m, 2H), 8.14 – 8.17 (m, 2H), 9.57 (s, 1H), 11.17 (s, 1H); ¹³C NMR (151 MHz, DMSO-*d*₆) δ 118.51, 124.95, 127.47, 129.09, 133.51, 139.65, 142.12, 144.55, 149.39; **LC-MS(ESI)** (90% H₂O + 2 mM NH₄Ac to 100% MeOH + 2 mM NH₄Ac in 10 min, then 100% MeOH + 2 mM NH₄Ac to 20 min, DAD 220-400 nm), *t*_R = 7.41 min, 98% purity, *m/z* [M+H]⁺ calcd for C₁₃H₁₁N₃O₅S 322.1, found 322.2; **HRMS(ESI)** *m/z* [M-H]⁻ calcd for C₁₃H₁₀N₃O₅S 320.0347, found 320.0347.



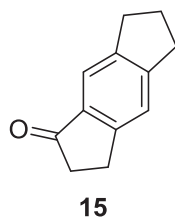
N-(Cyclohexylcarbamoyl)benzenesulfonamide (12). Benzenesulfonamide (472 mg, 3 mmol) was dissolved in dry THF (15 mL). NaH (60 % dispersion in mineral oil, 216 mg, 5.4 mmol) was added and the reaction mixture was stirred at 0 °C under argon for 30 min. Cyclohexyl isocyanate (376 mg, 3 mmol) was dissolved in dry THF (7.5 mL) and added in one portion. The reaction mixture stirred for 4 h at room temperature under argon. The reaction was quenched by dropwise adding of H₂O (1 mL) and the organic solvent was removed under reduced pressure. The residue was partitioned between EtOAc (100 mL) and saturated NH₄Cl solution (100 mL). The organic layer was separated. The aqueous phase was extracted with EtOAc (2 × 50 mL). The combined organic layers were dried over Na₂SO₄, filtered and concentrated to yield **12** as a white solid (813 mg).

Yield 96%; mp: 190-192 °C, lit.⁸ mp: 188-189 °C; ¹H NMR (500 MHz, DMSO-*d*₆) δ 1.05 – 1.16 (m, 4H), 1.19 – 1.24 (m, 2H), 1.46 – 1.51 (m, 1H), 1.55 – 1.60 (m, 2H), 1.62 – 1.67 (m, 2H), 6.33 (d, *J* = 7.9 Hz, 1H), 7.58 – 7.63 (m, 2H), 7.65 – 7.70 (m, 1H), 7.88 – 7.91 (m, 2H), 10.32 (s, 1H); ¹³C NMR (126 MHz, DMSO-*d*₆) δ 24.10, 24.92, 32.20, 48.05, 127.09, 128.93, 133.07, 140.25, 150.37; **LC-MS(ESI)** (90% H₂O + 2 mM NH₄Ac to 100% MeOH + 2 mM NH₄Ac in 10 min, then 100% MeOH + 2 mM NH₄Ac to 20 min, DAD 200-400 nm), *t*_R = 9.21 min, 99% purity, *m/z* [M+H]⁺ calcd for C₁₃H₁₈N₂O₃S 283.1, found 283.0; **HRMS(ESI)** *m/z* [M+Na]⁺ calcd for C₁₃H₁₈N₂O₃S 305.0930, found 305.0942.



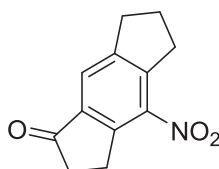
3-Chloro-1-(2,3-dihydro-1H-inden-5-yl)propan-1-one (14). AlCl₃ (2.9 g, 22 mmol) was dispersed in dry CH₂Cl₂ (20 mL) and cooled to -10 °C under nitrogen. Indane (**13**, 2.4 g, 20 mmol) and 3-chloropropionyl chloride (2.5 g, 20 mmol) were dissolved in dry CH₂Cl₂ (20 mL) and added dropwise over 1 h. The mixture was allowed to warm to room temperature and was stirred for 18 h. The solution was carefully added to 2 N HCl (25 mL) at -10 °C. The organic layer was separated. The aqueous phase was extracted with CH₂Cl₂ (3 × 50 mL). The combined organic layers were washed with saturated sodium bicarbonate solution (50 mL) and brine (50 mL), dried over Na₂SO₄, filtered and concentrated to yield **14** as a beige solid (3.65 g).

Yield 88%; mp: 66-68 °C, lit.¹ mp: 64-66 °C; ¹H NMR (500 MHz, DMSO-*d*₆) δ 2.05 (quint, *J* = 7.5 Hz, 2H), 2.92 (t, *J* = 7.5 Hz, 4H), 3.50 (t, *J* = 6.3 Hz, 2H), 3.91 (t, *J* = 6.3 Hz, 2H), 7.36 (d, *J* = 7.8 Hz, 1H), 7.73 – 7.79 (m, 1H), 7.83 (s, 1H); ¹³C NMR (126 MHz, DMSO-*d*₆) δ 24.90, 31.95, 32.40, 39.60, 40.63, 123.76, 124.37, 126.45, 134.81, 144.43, 150.05, 196.56; LC-MS(ESI) (90% H₂O + 2 mM NH₄Ac to 100% MeOH + 2 mM NH₄Ac in 10 min, then 100% MeOH + 2 mM NH₄Ac to 20 min, DAD 220-400 nm), *t*_R = 11.65 min, 99% purity, *m/z* [M+H]⁺ calcd for C₁₂H₁₃ClO 209.1, found 209.0.



3,5,6,7-Tetrahydro-s-indacen-1(2H)-one (15). 3-Chloro-1-(2,3-dihydro-1H-inden-5-yl)propan-1-one (**14**, 2.9 g, 14 mmol) was added under stirring to concentrated H₂SO₄ (20 mL). The mixture was stirred for 48 h at 55 °C. The mixture was slowly poured onto crushed ice and the built precipitate was collected. The crude brown product was purified by silica gel flash column chromatography using a gradient of 100% petroleum ether to 25% EtOAc as eluent to yield **15** as a light beige solid (2.06 g).

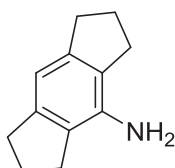
Yield 85%; mp: 80-82 °C, lit.⁹ mp: 80-81 °C; ¹H NMR (500 MHz, DMSO-*d*₆) δ 2.05 (quint, *J* = 7.4 Hz, 2H), 2.58 – 2.64 (m, 2H), 2.87 (t, *J* = 7.4 Hz, 2H), 2.91 (t, *J* = 7.4 Hz, 2H), 3.00 – 3.04 (m, 2H), 7.38 (s, 1H), 7.43 (s, 1H); ¹³C NMR (126 MHz, DMSO-*d*₆) δ 25.03, 25.35, 31.43, 32.43, 36.30, 118.01, 122.32, 135.48, 143.54, 152.32, 154.28, 205.47; LC-MS(ESI) (90% H₂O with 2 mM NH₄Ac to 100% MeCN in 10 min, then 100% MeCN to 15 min, DAD 200-600 nm), *t*_R = 6.05 min, 100% purity, *m/z* [M+H]⁺ calcd for C₁₂H₁₂O 173.1, found 173.1.



16

4-Nitro-3,5,6,7-tetrahydro-s-indacen-1(2H)-one (16). 3,5,6,7-Tetrahydro-s-indacen-1(2H)-one (**15**, 517 mg, 3 mmol) was dissolved in dry CH₂Cl₂ (12 mL), acetic anhydride (8 mL) and acetic acid (6 mL) at 0 °C. Fuming nitric acid (1.2 mL) was added dropwise over 2 h at 0 °C. The reaction mixture was allowed to stir for 1 h at 0 °C. The solution was poured onto crushed ice and was extracted with CH₂Cl₂ (3 × 50 mL). The combined organic layers were washed with saturated NaHCO₃ solution (50 mL) and brine (50 mL), dried over Na₂SO₄, filtered and concentrated. The crude product was purified by silica gel column chromatography using petroleum ether / EtOAc (4+1) as eluent to yield **16** as a yellow resin (276 mg).

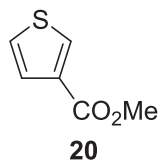
Yield 42%; lit.¹ mp: 99-101 °C; ¹H NMR (500 MHz, DMSO-*d*₆) δ 2.13 (quint, *J* = 7.6 Hz, 2H), 2.70 – 2.75 (m, 2H), 2.89 (t, *J* = 7.5 Hz, 2H), 3.03 (t, *J* = 7.5 Hz, 2H), 3.08 – 3.13 (m, 2H), 7.66 (s, 1H); ¹³C NMR (126 MHz, DMSO-*d*₆) δ 24.96, 25.10, 28.81, 32.78, 36.81, 125.34, 125.47, 135.64, 155.47, 156.67, 201.03. One aromatic ¹³C signal is missing. **LC-MS(ESI)** (90% H₂O + 2 mM NH₄Ac to 100% MeCN in 10 min, then 100% MeCN to 15 min, DAD 200-600 nm), *t*_R = 6.08 min, 99% purity, *m/z* [M+H]⁺ calcd for C₁₂H₁₁NO₃ 218.1, found 218.1.



17

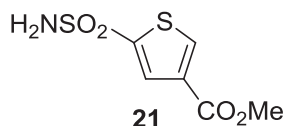
1,2,3,5,6,7-Hexahydro-s-indacen-4-amine (17). Pd(OH)₂ on carbon (Pearlman's catalyst, 50% water wet, 55 mg) was placed in a Schlenk flask under H₂. A mixture of 4-nitro-3,5,6,7-tetrahydro-s-indacen-1(2H)-one (**16**, 217 mg, 1 mmol) and methanesulfonic acid (1.06 g, 1.1 mmol) was added by syringe and the mixture was allowed to stir for 18 h at room temperature under H₂ (1 atm, balloon). The reaction mixture was filtered through celite and the filtrate was evaporated. The crude product was purified by silica gel column chromatography using petroleum ether / EtOAc (1+1) + 1% TEA as eluent to yield **17** as a colourless solid (96 mg).

Yield 55%; mp: 88-90 °C, lit.¹ mp: 88-89 °C; ¹H NMR (500 MHz, DMSO-*d*₆) δ 1.96 (quint, *J* = 7.4 Hz, 4H), 2.58 (t, *J* = 7.3 Hz, 4H), 2.71 (t, *J* = 7.4 Hz, 4H), 4.48 (s, 2H), 6.34 (s, 1H); ¹³C NMR (126 MHz, DMSO-*d*₆) δ 24.90, 28.85, 32.57, 108.27, 124.72, 139.59, 142.43; **LC-MS(ESI)** (90% H₂O + 2 mM NH₄Ac to 100% MeCN in 10 min, then 100% MeCN to 15 min, DAD 200-600 nm), *t*_R = 6.90 min, 98% purity, *m/z* [M+H]⁺ calcd for C₁₂H₁₅N 174.1, found 174.1.



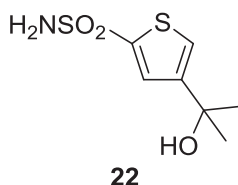
Methyl thiophene-3-carboxylate (20). Thiophene-3-carboxylic acid (**19**, 1.92 g, 15 mmol) was dissolved in MeOH (30 mL). Concentrated H₂SO₄ (1.5 mL) was added and the mixture was refluxed for 18 h. The reaction mixture was concentrated and neutralized with saturated NaHCO₃ solution (20 mL). The aqueous phase was extracted with EtOAc (3 × 50 mL), dried over Na₂SO₄ and concentrated to yield **20** as a light-yellow liquid (2.07 g).

Yield 97%; ¹H NMR (500 MHz, DMSO-*d*₆) δ 3.80 (s, 3H), 7.46 (dd, *J* = 5.1, 1.3 Hz, 1H), 7.65 (dd, *J* = 5.1, 3.0 Hz, 1H), 8.34 (dd, *J* = 3.1, 1.3 Hz, 1H), lit.¹⁰ ¹H NMR (500 MHz, CDCl₃) δ 3.86 (s, 3H), 7.30 (dd, *J* = 5.1, 3.2 Hz, 1H), 7.52 (dd, *J* = 5.1, 1.2 Hz, 1H), 8.10 (dd, *J* = 3.2, 1.2 Hz, 1H); ¹³C NMR (126 MHz, DMSO-*d*₆) δ 51.65, 127.32, 127.62, 132.70, 133.67, 162.48; **LC-MS(ESI)** (90% H₂O + 2 mM NH₄Ac to 100% MeOH + 2 mM NH₄Ac in 10 min, then 100% MeOH + 2 mM NH₄Ac to 20 min, DAD 220-400 nm), *t*_R = 8.56 min, 100% purity, *m/z* [M+H]⁺ calcd for C₆H₆O₂S 143.0, found 143.0.



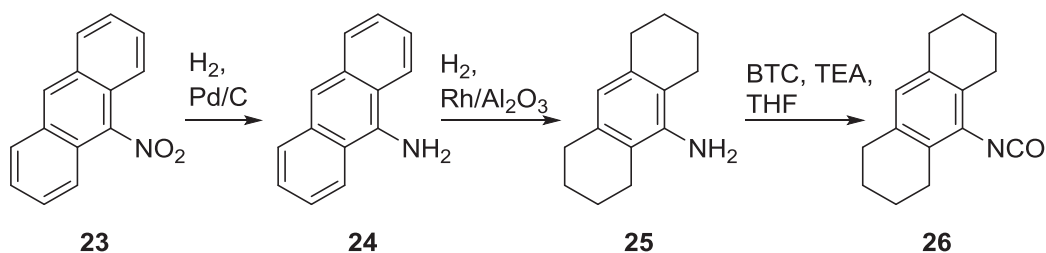
Methyl 5-sulfamoylthiophene-3-carboxylate (21). Chlorosulfonic acid (10.5 g, 90 mmol) was stirred at 0 °C under argon atmosphere. Methyl thiophene-3-carboxylate (**20**, 2.13 g, 15 mmol) was added dropwise at 0 °C over 30 min. The reaction mixture was heated to 60 °C for 3 h. The solution was slowly poured onto crushed ice and the aqueous phase was extracted with CH₂Cl₂ (3 × 50 mL). The combined organic phases were dried over Na₂SO₄, filtered and concentrated. The crude intermediate methyl 5-(chlorosulfonyl)thiophene-3-carboxylate was dissolved in dry MeCN (30 mL) and ammonia gas was added (1 atm, balloon). The reaction mixture was stirred for 1 h at room temperature. The organic phase was evaporated. The precipitate was collected, washed with water (20 mL) and hexane (20 mL). The product was dried under high vacuum to yield **21** as a white solid (510 mg).

Yield 15%; mp: 128-130 °C; ¹H NMR (600 MHz, DMSO-*d*₆) δ 3.83 (s, 3H), 7.81 – 7.82 (m, 3H), 8.55 (d, *J* = 1.5 Hz, 1H); ¹³C NMR (151 MHz, DMSO-*d*₆) δ 52.14, 129.19, 132.01, 137.47, 146.98, 161.65; **LC-MS(ESI)** (90% H₂O + 2 mM NH₄Ac to 100% MeOH + 2 mM NH₄Ac in 10 min, then 100% MeOH + 2 mM NH₄Ac to 20 min, DAD 210-400 nm), *t*_R = 5.71 min, 99% purity, *m/z* [M+H]⁺ calcd for C₆H₇NO₄S₂ 222.0, found 221.9.

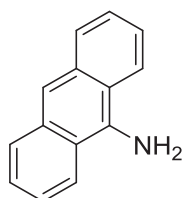


4-(2-Hydroxypropan-2-yl)thiophene-2-sulfonamide (22). Methyl 5-sulfamoylthiophene-3-carboxylate (**21**, 487 mg, 2.2 mmol) was dissolved in dry THF (10 mL) and placed under nitrogen atmosphere. The solution was cooled to 0 °C. CH₃MgBr (3 M in Et₂O, 7.33 mL, 22 mmol) was added carefully. The reaction mixture was allowed to stir at room temperature for 2 h. The reaction was quenched with saturated NH₄Cl solution (50 mL). The aqueous phase was extracted with CH₂Cl₂ (3 × 30 mL). The combined organic layers were washed with brine (50 mL), dried over Na₂SO₄ and concentrated. The crude product was purified by silica gel flash column chromatography using a gradient from petroleum ether to petroleum ether / EtOAc (1+1) to yield **22** as a white solid (413 mg).

Yield 85%; mp: 108-110 °C; ¹H NMR (600 MHz, DMSO-*d*₆) δ 1.42 (s, 6H), 5.19 (s, 1H), 7.52 (d, *J* = 1.7 Hz, 1H), 7.55 (d, *J* = 1.7 Hz, 1H), 7.58 (s, 2H); ¹³C NMR (151 MHz, DMSO-*d*₆) δ 31.39, 69.30, 123.61, 128.84, 145.04, 152.36; **LC-MS(ESI)** (90% H₂O + 2 mM NH₄Ac to 100% MeOH + 2 mM NH₄Ac in 10 min, then 100% MeOH + 2 mM NH₄Ac to 20 min, DAD 220-400 nm), *t*_R = 4.62 min, 99% purity, *m/z* [M+NH₄]⁺ calcd for C₇H₁₁NO₃S₂ 239.1, found 238.9.



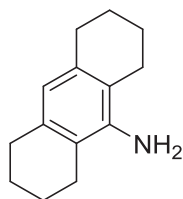
Scheme S2. Synthesis of 1,2,3,4,5,6,7,8-octahydroanthracen-9-amine 25



24

Anthracen-9-amine (24). 9-Nitroanthracene (**23**, 893 mg, 4 mmol) was dispersed in dry EtOH (20 mL) and treated with 10% Pd/C (89 mg) under H₂ (1 atm, balloon) for 24 h. The reaction mixture was filtered through celite and was concentrated. The crude product was purified by silica gel flash column chromatography using a gradient from 100% CH₂Cl₂ to 3% MeOH to yield **24** as a yellow solid (360 mg).

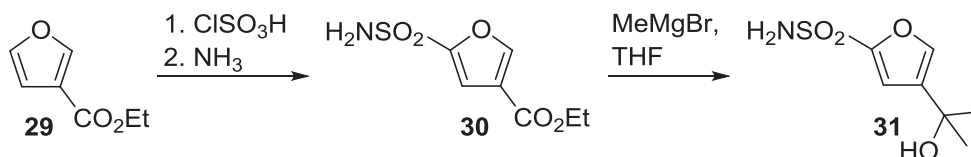
Yield 47%; mp: 144-146 °C, lit.¹¹ mp: 148-151 °C; ¹H NMR (500 MHz, DMSO-*d*₆) δ 6.58 (s, 2H), 7.27 – 7.31 (m, 2H), 7.37 – 7.41 (m, 2H), 7.63 (s, 1H), 7.84 (d, *J* = 8.4 Hz, 2H), 8.34 (d, *J* = 8.8 Hz, 2H); ¹³C NMR (126 MHz, DMSO-*d*₆) δ 111.96, 116.68, 122.08, 123.08, 125.23, 128.00, 132.13, 141.19; LC-MS(ESI) (90% H₂O + 2 mM NH₄Ac to 100% MeCN in 10 min, then 100% MeCN to 15 min, DAD 200-600 nm), *t*_R = 6.74 min, 88% purity, *m/z* [M+H]⁺ calcd for C₁₄H₁₁N 194.1, found 194.1.



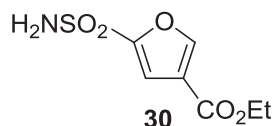
25

1,2,3,4,5,6,7,8-Octahydroanthracen-9-amine (25). Anthracen-9-amine (**24**, 348 mg, 1.8 mmol) was dissolved in dry EtOH (15 mL) and treated with Rh 5% on activated Al₂O₃ under H₂ for 48 h. The suspension was filtered through celite and was concentrated. The crude product was purified by silica gel flash column chromatography using a gradient of petroleum ether / EtOAc (4+1) to petroleum ether / EtOAc (1+1) to yield **25** as a white solid (313 mg).

Yield 86%; mp: 84-86 °C, lit.¹² mp: 83-84 °C; ¹H NMR (500 MHz, DMSO-*d*₆) δ 1.59 – 1.64 (m, 4H), 1.70 – 1.76 (m, 4H), 2.33 (t, *J* = 6.5 Hz, 4H), 2.54 (t, *J* = 6.2 Hz, 4H), 4.24 (s, 2H), 6.06 (s, 1H), ¹³C NMR (126 MHz, DMSO-*d*₆) δ 22.68, 23.05, 23.77, 29.37, 116.96, 117.55, 133.26, 142.83; LC-MS(ESI) (90% H₂O + 2 mM NH₄Ac to 100% MeCN in 10 min, then 100% MeCN to 15 min, DAD 200-600 nm), *t*_R = 7.91 min, 95% purity, *m/z* [M+H]⁺ calcd for C₁₄H₁₉N 202.2, found 202.1.

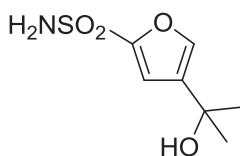


Scheme S3. Synthesis of the sulfonamide building block 31 for sulfonylurea 1



Ethyl 5-sulfamoylfuran-3-carboxylate (30). This compound was synthesized as described elsewhere.¹³ 3-Ethyl furoate (**29**, 1.45 mL, 10.7 mmol) was dissolved in dry CH₂Cl₂ (20 mL) under nitrogen. The solution was cooled to -30 °C and chlorosulfonic acid (0.75 mL, 11.5 mmol) was added dropwise over 10 min at -30 °C. The reaction was allowed to warm to room temperature and was stirred for 72 h. The mixture was cooled to -10 °C and dry pyridine (0.95 mL, 11.7 mmol) was added slowly over 10 min. PCl₅ (2.45 g, 11.7 mmol) was added in portions at -10 °C. The reaction mixture was allowed to warm to room temperature and was stirred for 18 h. The reaction was quenched by dropwise addition to ice-cool water (50 mL) over 30 min. The mixture was extracted with CH₂Cl₂ (3 × 50 mL). The combined organic layers were washed with water (50 mL), dried over Na₂SO₄, filtered and concentrated to yield the crude intermediate ethyl 5-(chlorosulfonyl)furan-3-carboxylate. The crude intermediate was dissolved in dry THF (20 mL) and cooled to -78 °C. Ammonia gas was liquefied at -78 °C and 10 mL were added to the reaction at -78 °C. The reaction mixture was allowed to slowly warm to room temperature and was stirred for 18 h. The reaction mixture was concentrated under vacuum and the crude product was purified by silica gel flash column chromatography using a gradient of 100 % CH₂Cl₂ to 10% MeOH to yield **30** as a light beige solid (845 mg).

Yield 36%; mp: 130-132 °C, lit.¹ mp: 131-132.5 °C; ¹H NMR (500 MHz, DMSO-*d*₆) δ 1.26 – 1.30 (m, 3H), 4.24 – 4.29 (m, 2H), 7.11 – 7.13 (m, 1H), 7.96 (s, 2H), 8.63 – 8.64 (m, 1H), ¹³C NMR (126 MHz, DMSO-*d*₆) δ 14.12, 60.77, 111.58, 119.59, 150.40, 153.50, 161.33; LC-MS(ESI) (90% H₂O + 2 mM NH₄Ac to 100% MeCN in 10 min, then 100% MeCN to 15 min, DAD 200-600 nm), *t*_R = 2.76 min, 98% purity, *m/z* [M+NH₄]⁺ calcd for C₇H₉NO₅S 237.1, found 237.1.



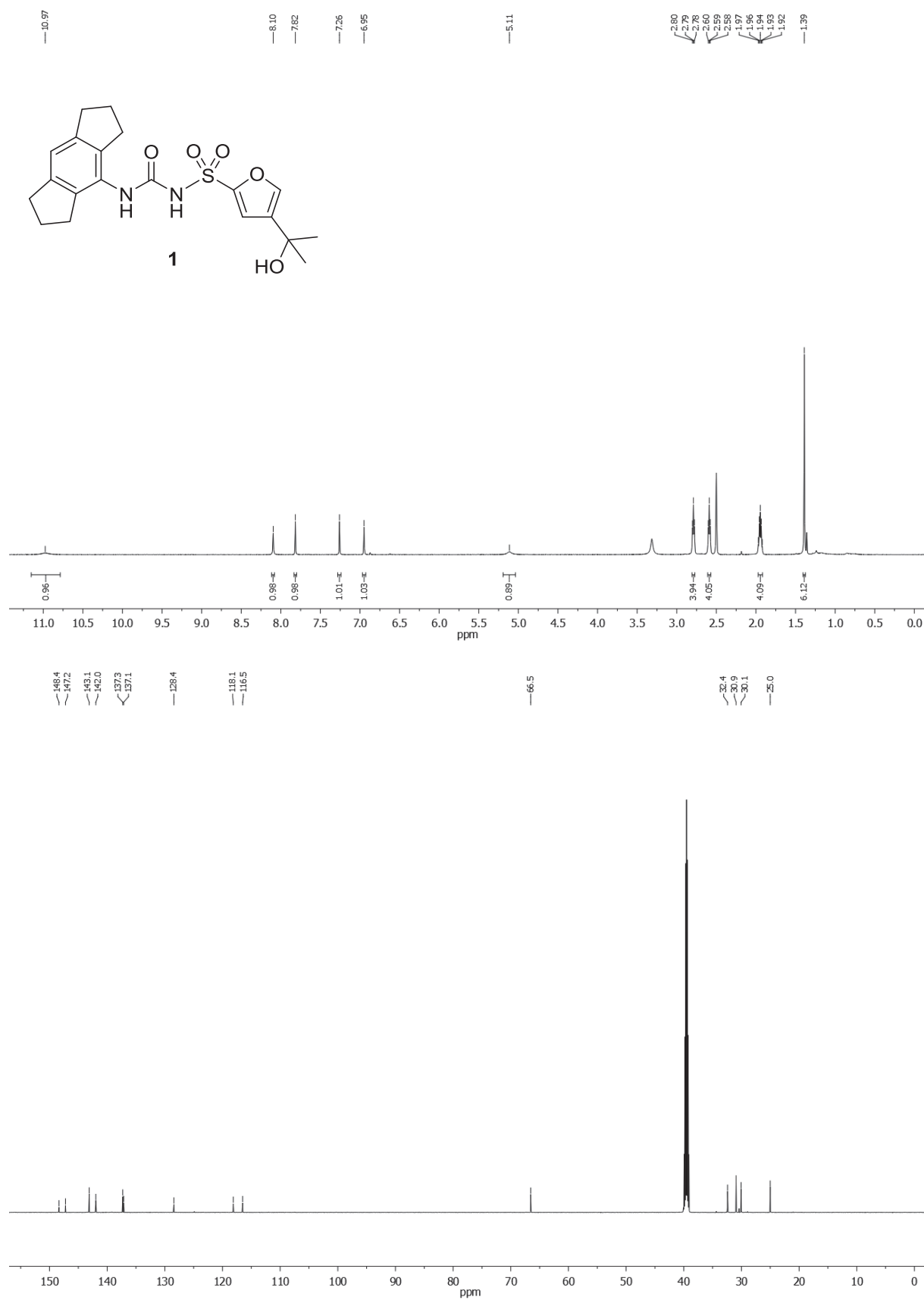
31

4-(2-Hydroxypropan-2-yl)furan-2-sulfonamide (31). Ethyl 5-sulfamoylfuran-3-carboxylate (**30**, 482 mg, 2.2 mmol) was dissolved in dry THF (10 mL) under nitrogen. The solution was cooled to 0 °C. CH_3MgBr (3 M solution in Et_2O , 7.33 mL, 22 mmol) was carefully added at 0 °C. The reaction mixture was allowed to warm to room temperature and stirred for 2 h. The reaction was quenched with saturated NH_4Cl solution (50 mL) and extracted with CH_2Cl_2 (3×50 mL). The combined organic layers were washed with brine (50 mL), dried over Na_2SO_4 , filtered and concentrated. The crude product was purified by silica gel flash column chromatography using a gradient of 100% CH_2Cl_2 to 10% MeOH to yield **31** as a beige solid (375 mg).

Yield 83%; mp: 100-102 °C; lit.¹ mp: 99.5-101.5 °C; $^1\text{H NMR}$ (500 MHz, $\text{DMSO}-d_6$) δ 1.38 – 1.39 (m, 6H), 5.08 (d, $J = 1.5$ Hz, 1H), 6.94 – 6.96 (m, 1H), 7.66 – 7.69 (m, 3H), $^{13}\text{C NMR}$ (126 MHz, $\text{DMSO}-d_6$) δ 30.97, 66.57, 112.11, 136.56, 140.26, 151.69; **LC-MS(ESI)** (90% H_2O + 2 mM NH_4Ac to 100% MeCN in 10 min, then 100% MeCN to 15 min, DAD 200-600 nm), $t_R = 0.84$ min, 100% purity, m/z $[\text{M}-\text{H}]^-$ calcd for $\text{C}_7\text{H}_{11}\text{NO}_4\text{S}$ 204.0, found 204.0.

NMR data

^1H and ^{13}C NMR spectra of compound **1**



Chemical structure of compound **2** is shown above the spectra.

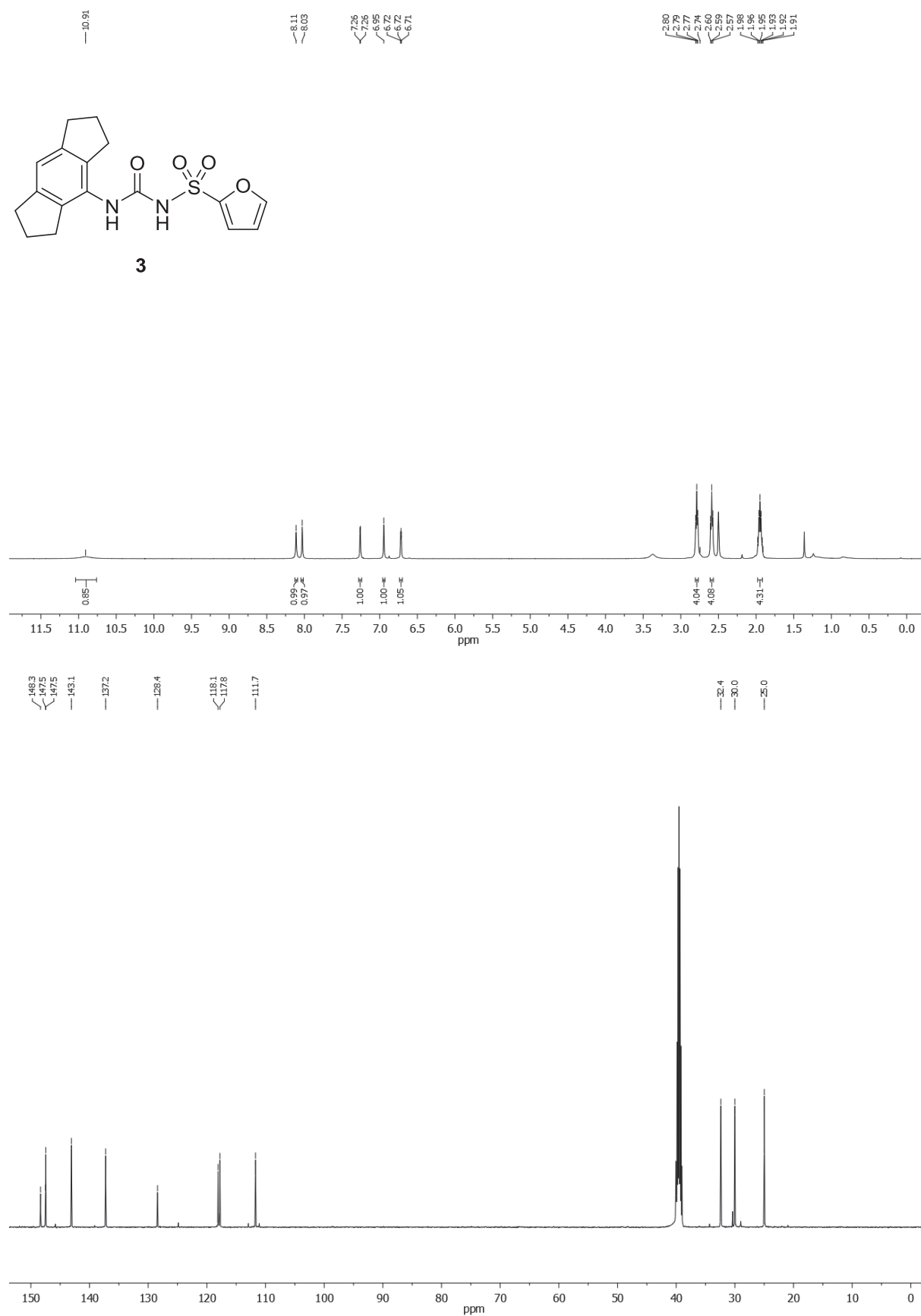
¹H NMR (CDCl₃) spectrum (top):

- Chemical shift range: 0.0 to 11.0 ppm.
- Integration values: 0.78, 0.93, 0.99, 0.97, 1.00, 0.92, 4.14, 4.16, 4.38, 6.02.
- Peak labels (ppm): 10.60, 8.10, 7.77, 7.76, 7.70, 7.70, 6.94, 5.23, 2.81, 2.79, 2.78, 2.69, 2.69, 2.57, 2.51, 2.50, 2.50, 2.49, 1.95, 1.95, 1.94, 1.92, 1.91.

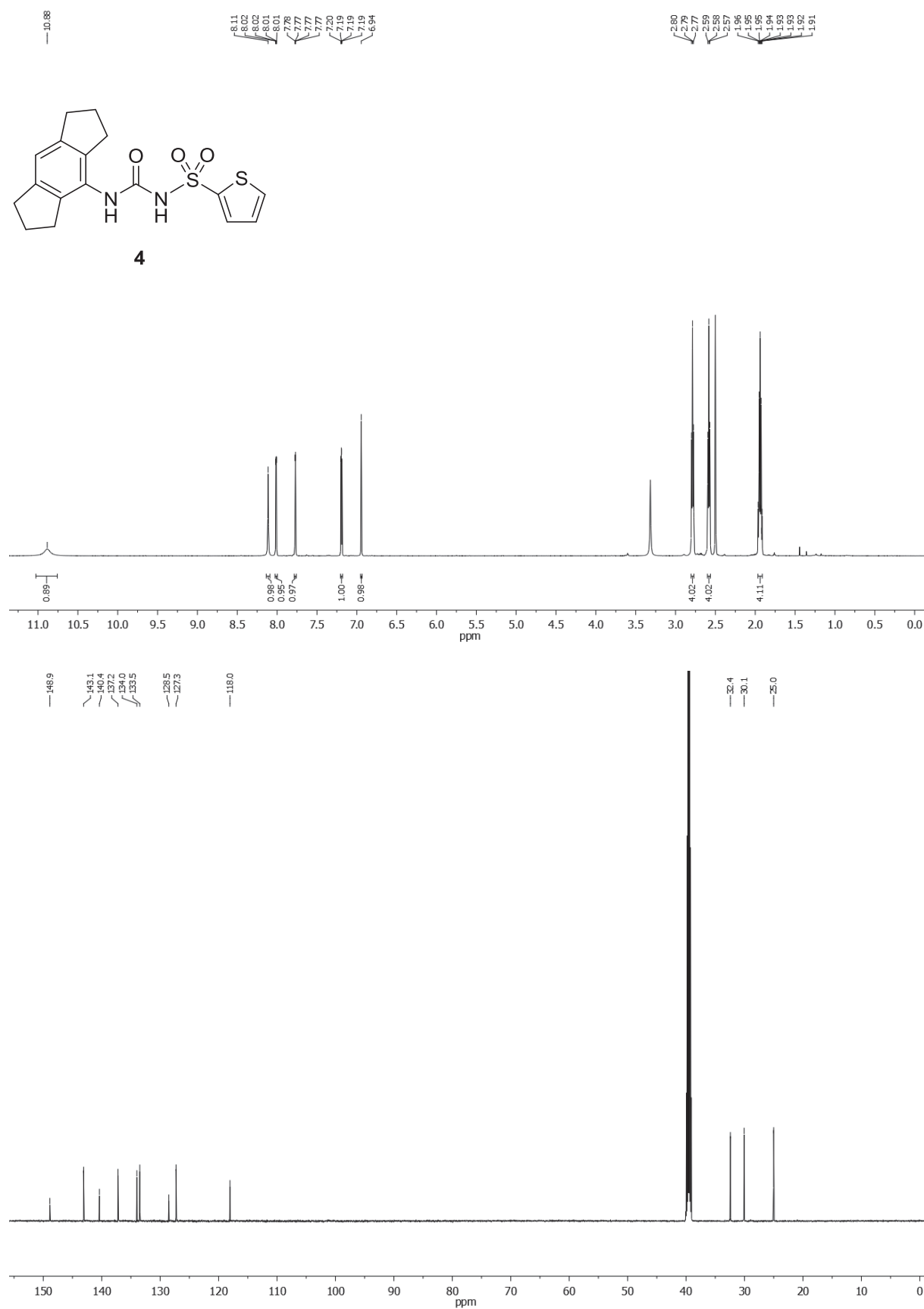
¹³C NMR (CDCl₃) spectrum (bottom):

- Chemical shift range: 0 to 160 ppm.
- Peak labels (ppm): 152.5, 148.8, 143.1, 139.9, 137.2, 131.9, 128.5, 126.5, 118.0, 69.3, 34.4, 33.3, 30.1, 25.0.

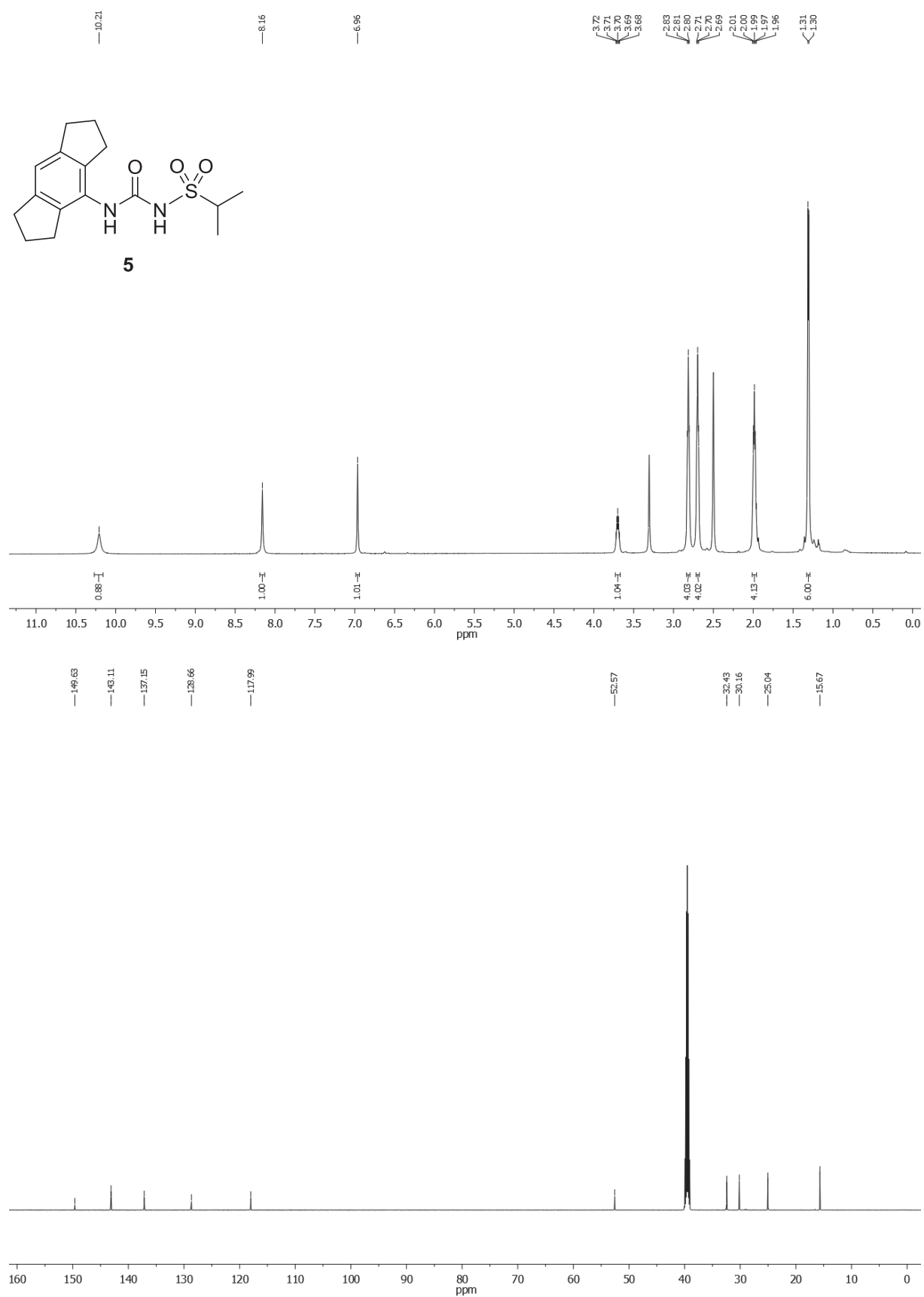
^1H and ^{13}C NMR spectra of compound **3**



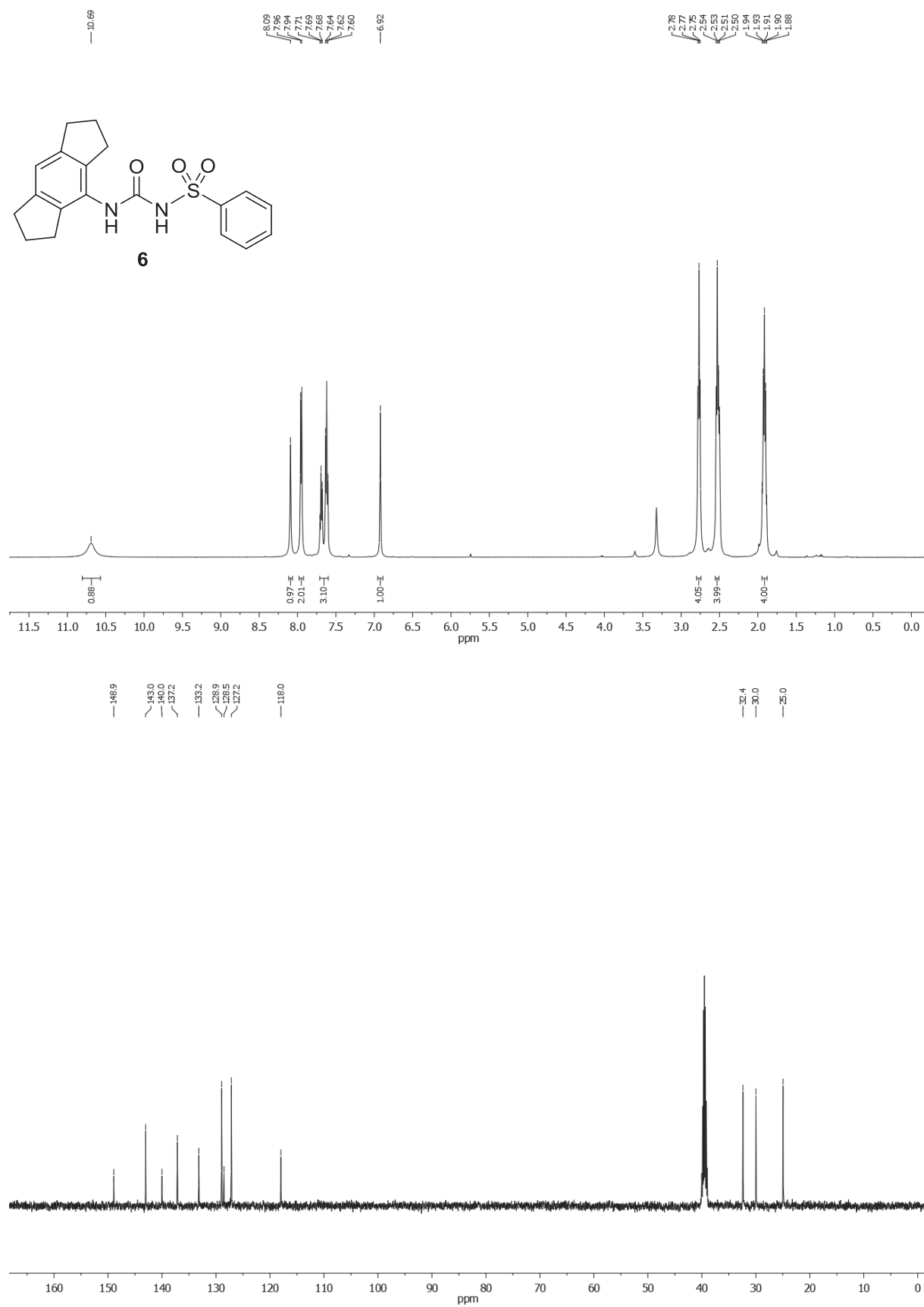
^1H and ^{13}C NMR spectra of compound **4**



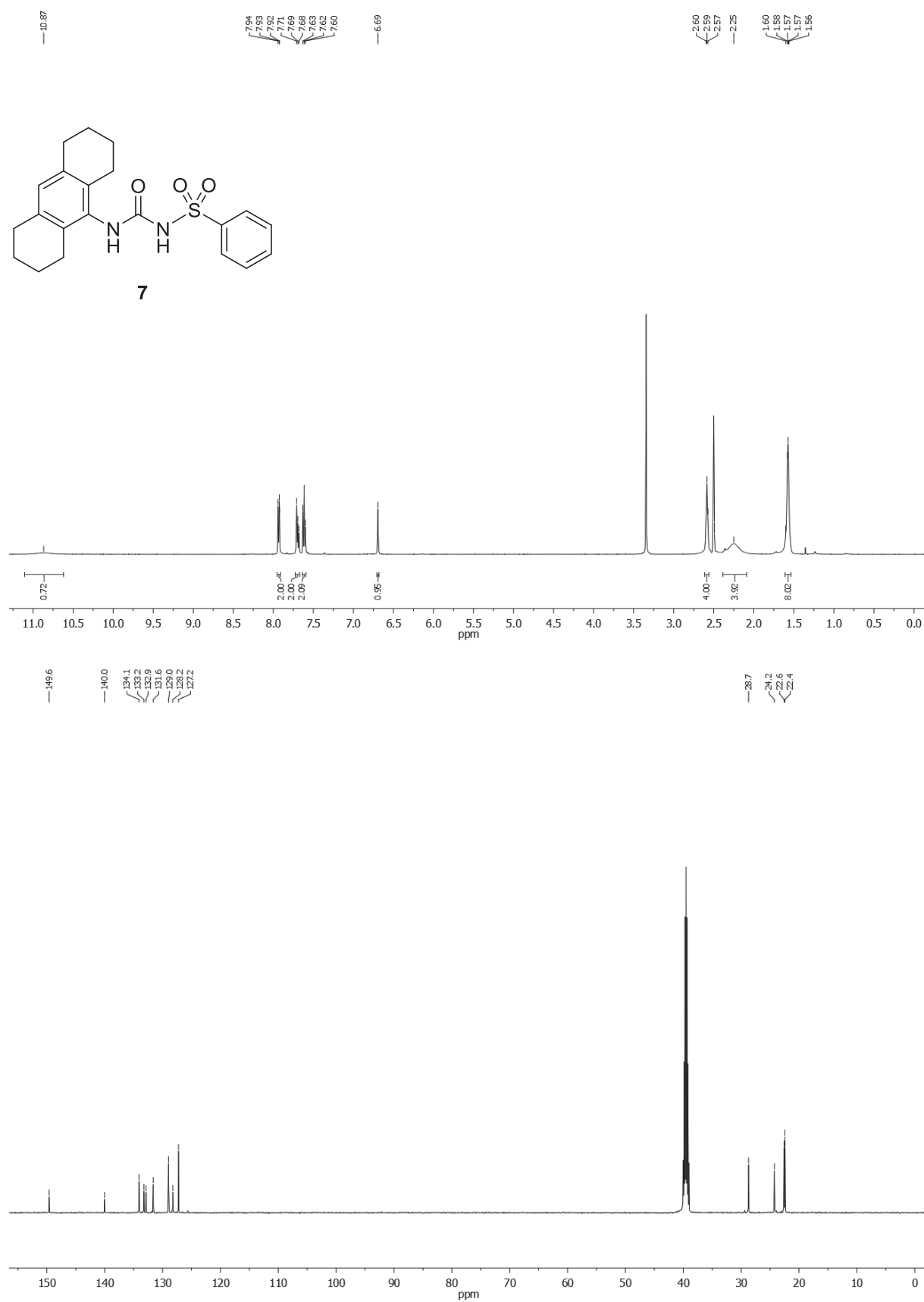
^1H and ^{13}C NMR spectra of compound **5**



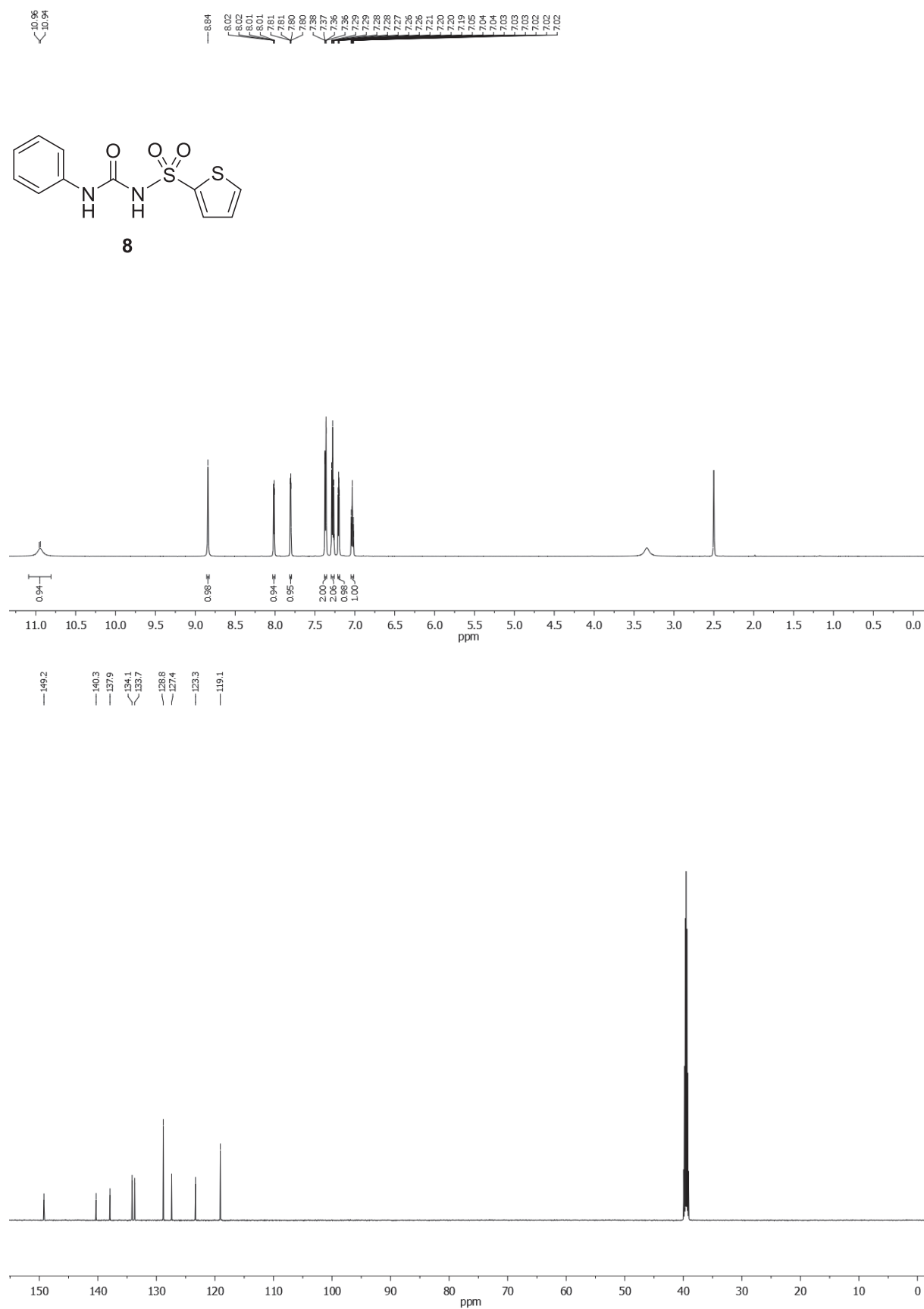
^1H and ^{13}C NMR spectra of compound **6**



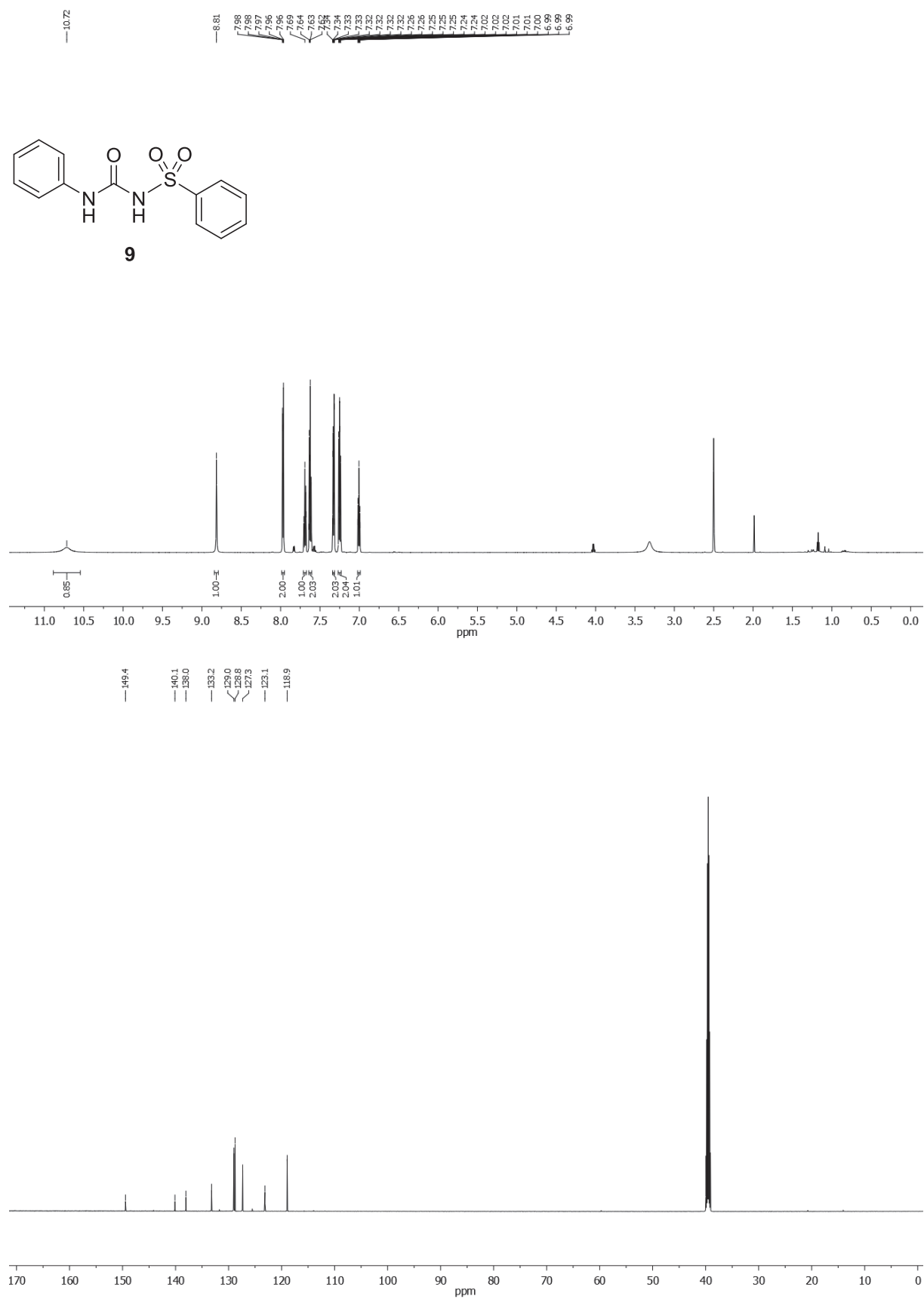
^1H and ^{13}C NMR spectra of compound **7**



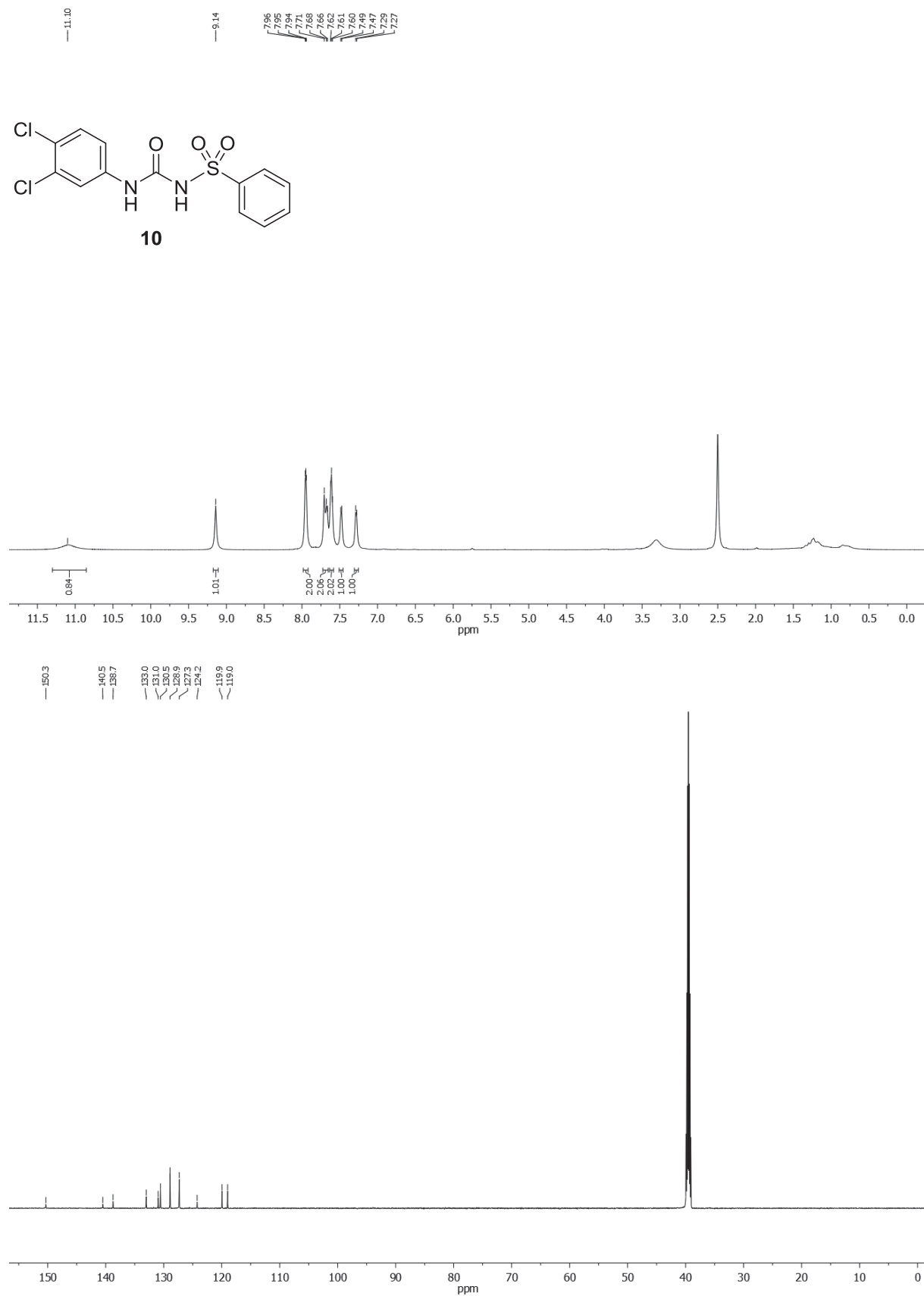
^1H and ^{13}C NMR spectra of compound **8**



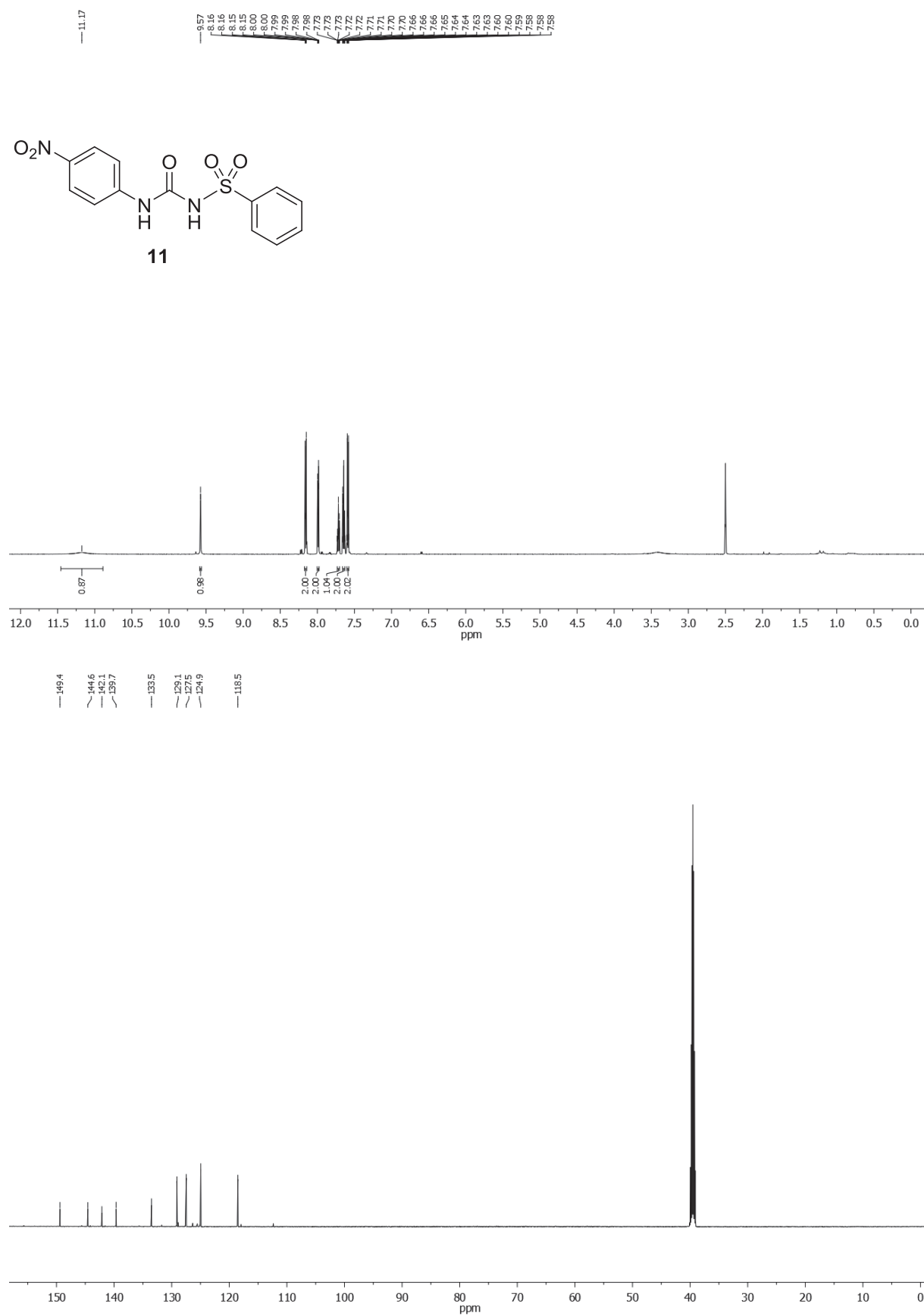
^1H and ^{13}C NMR spectra of compound **9**



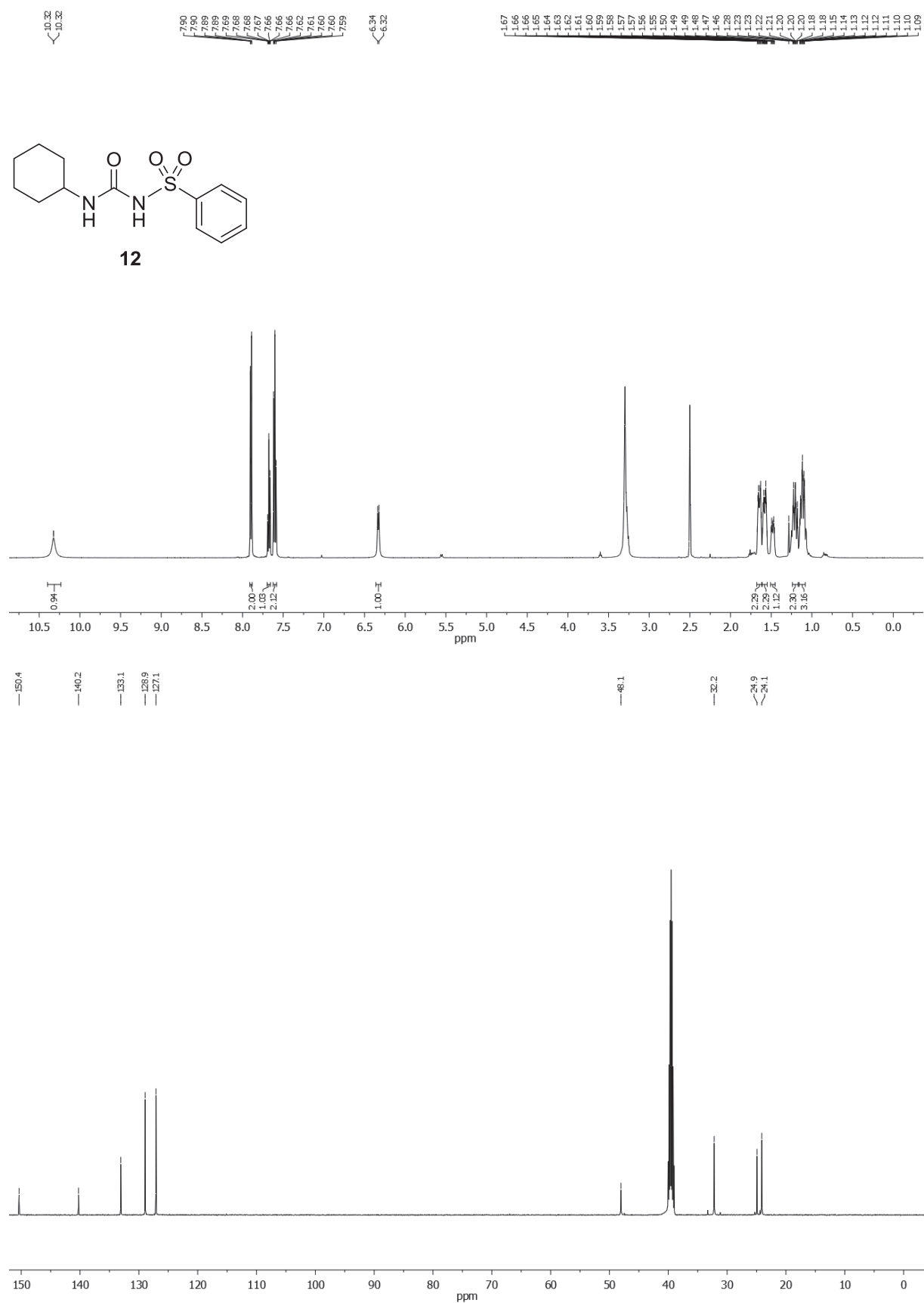
^1H and ^{13}C NMR spectra of compound **10**



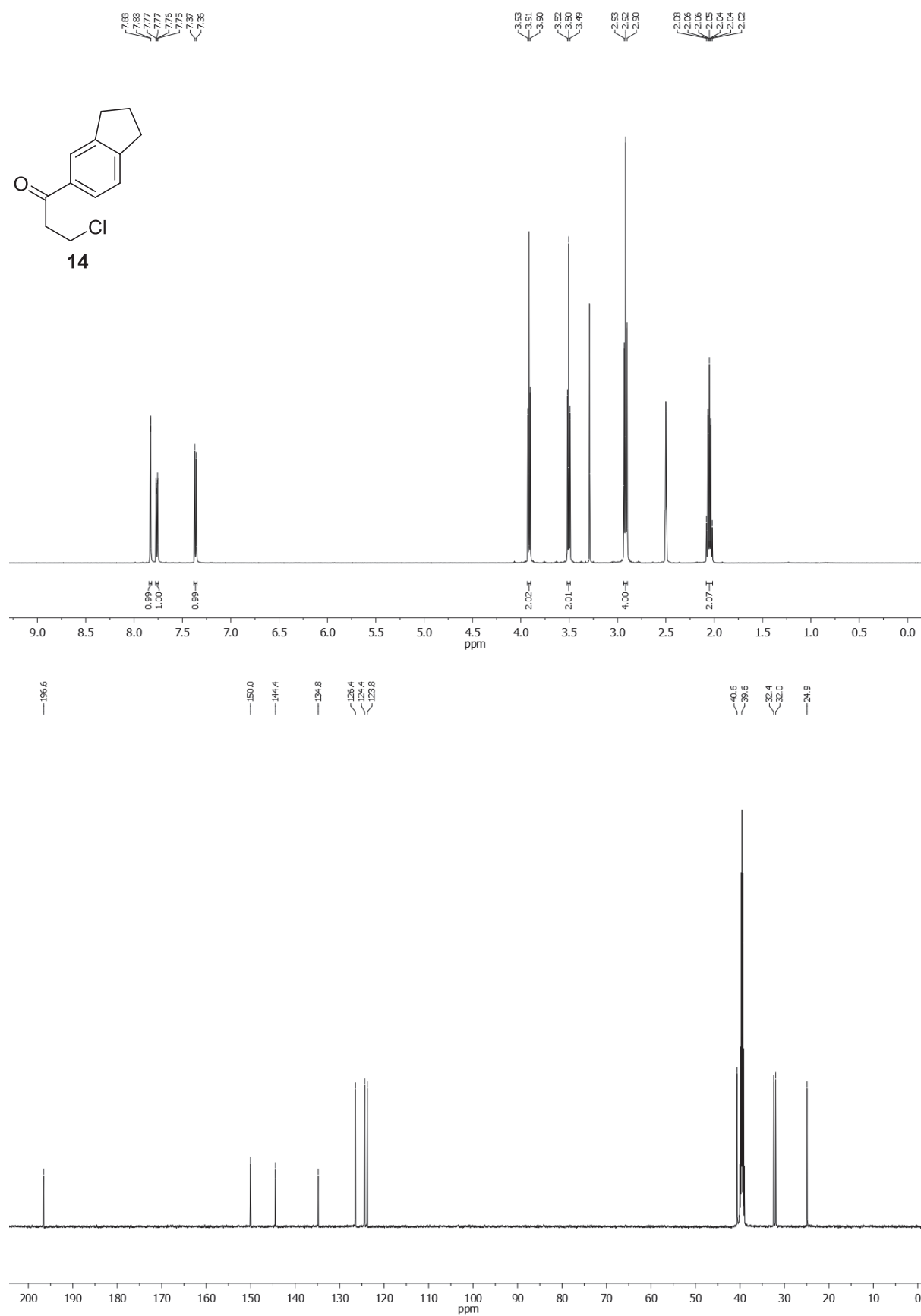
^1H and ^{13}C NMR spectra of compound **11**



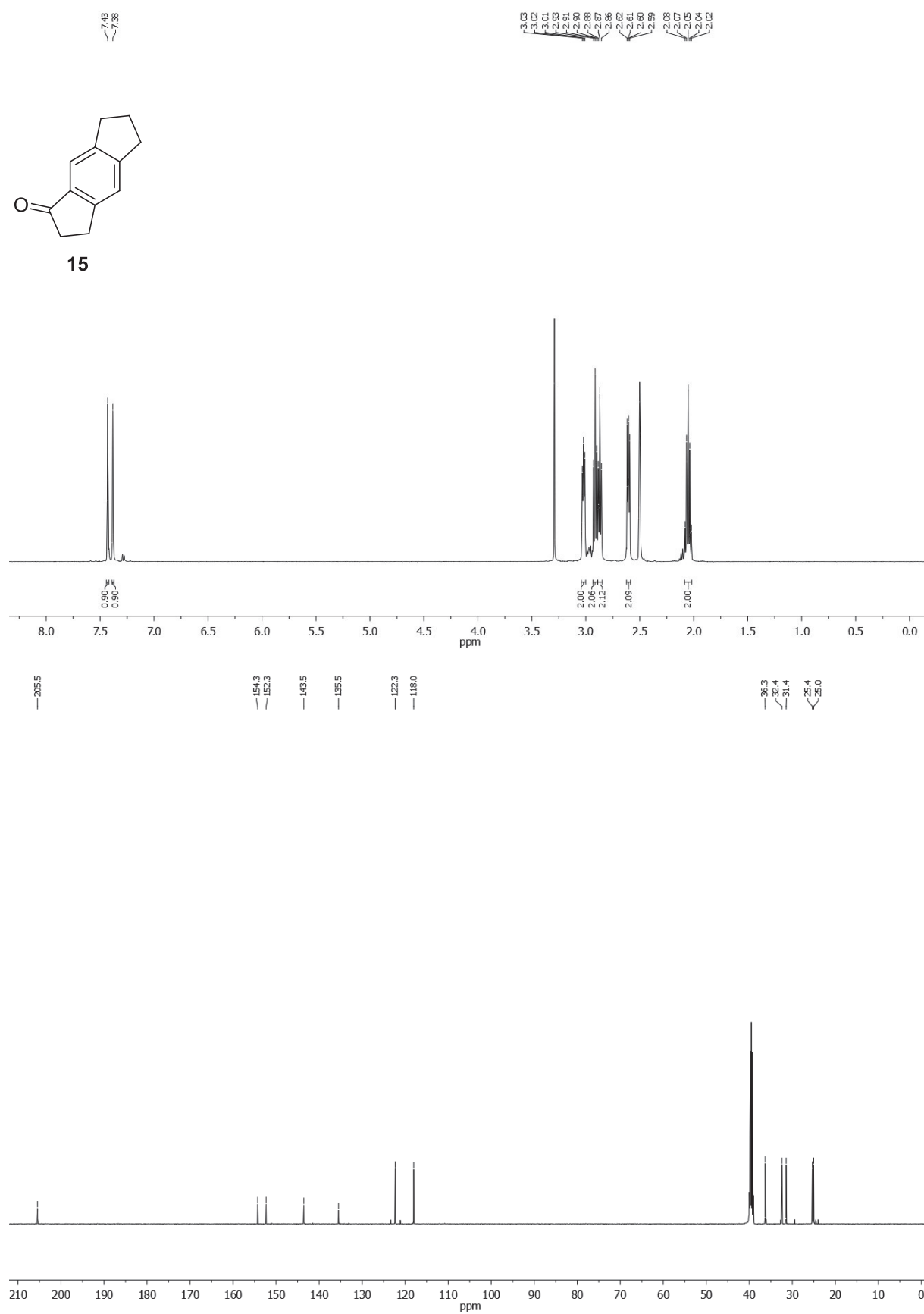
^1H and ^{13}C NMR spectra of compound **12**



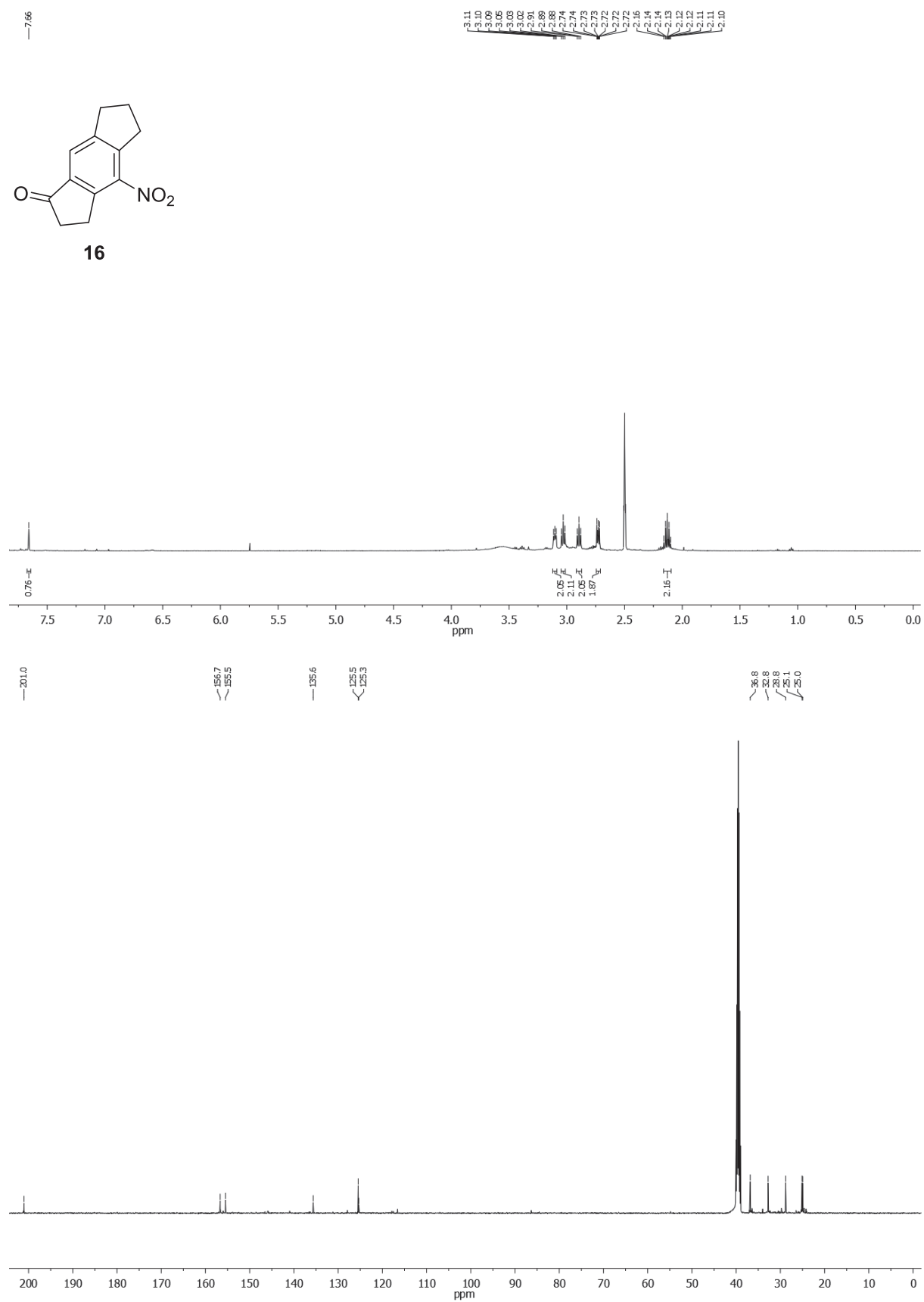
^1H and ^{13}C NMR spectra of compound **14**



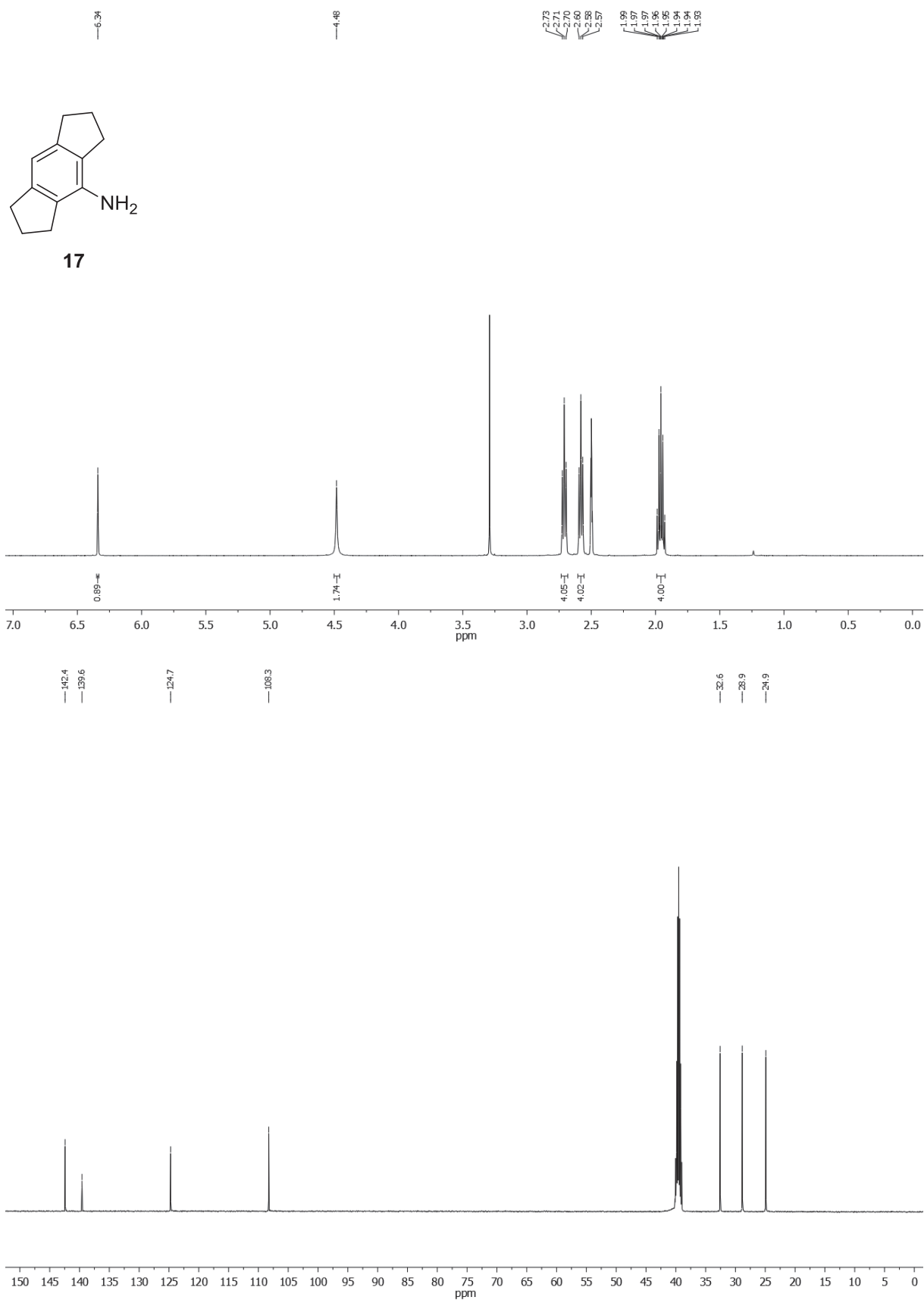
^1H and ^{13}C NMR spectra of compound **15**



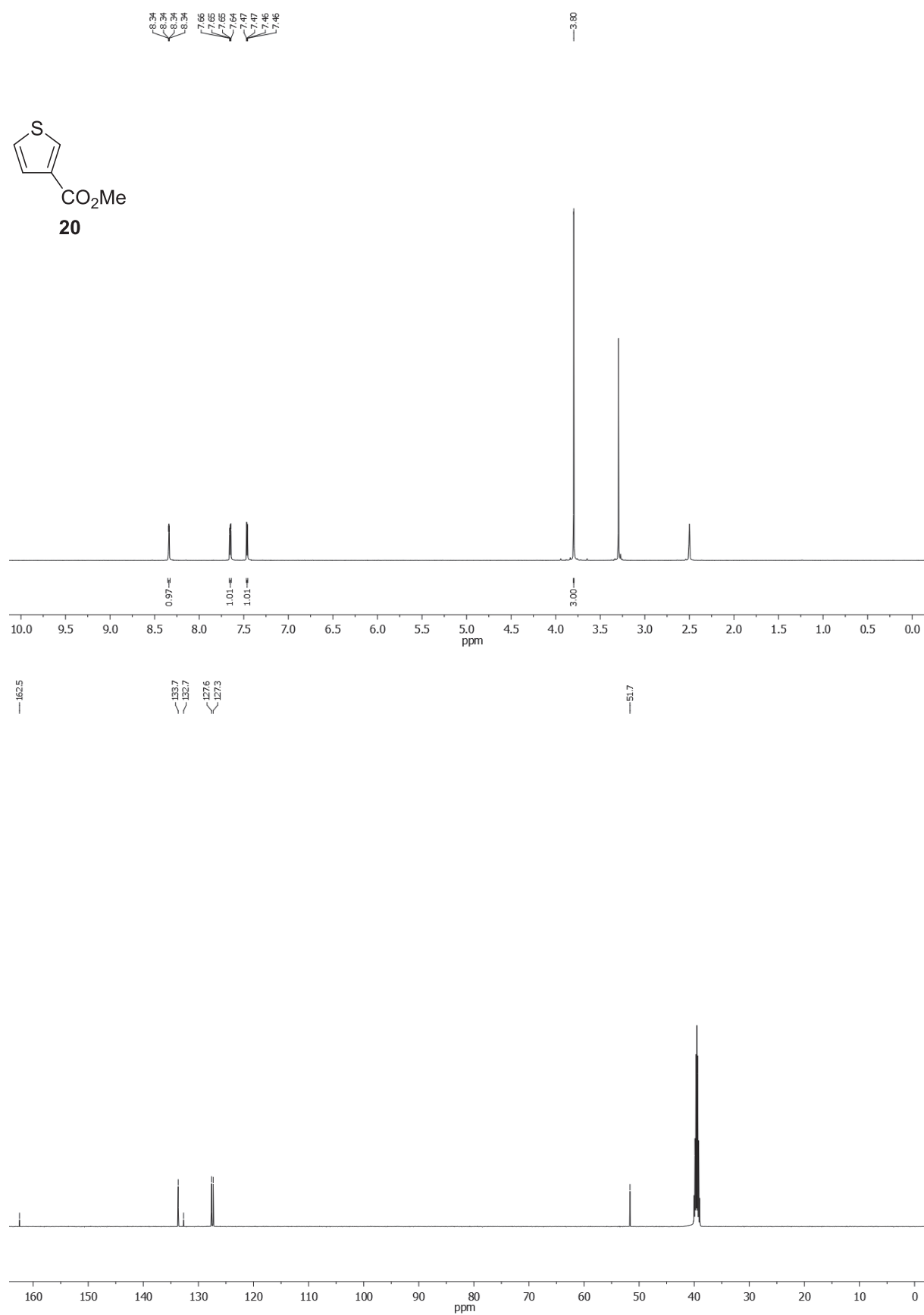
^1H and ^{13}C NMR spectra of compound **16**



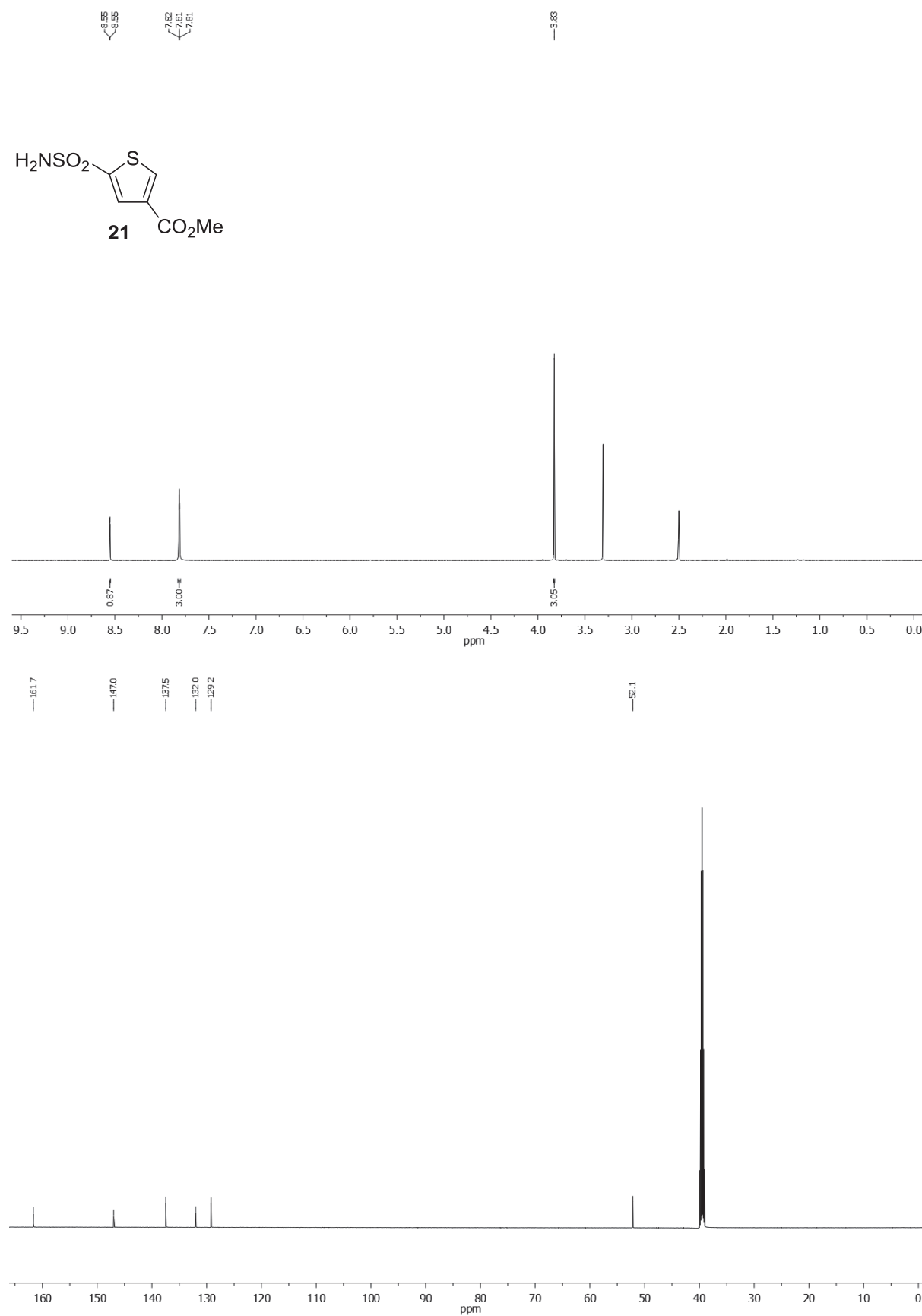
^1H and ^{13}C NMR spectra of compound **17**



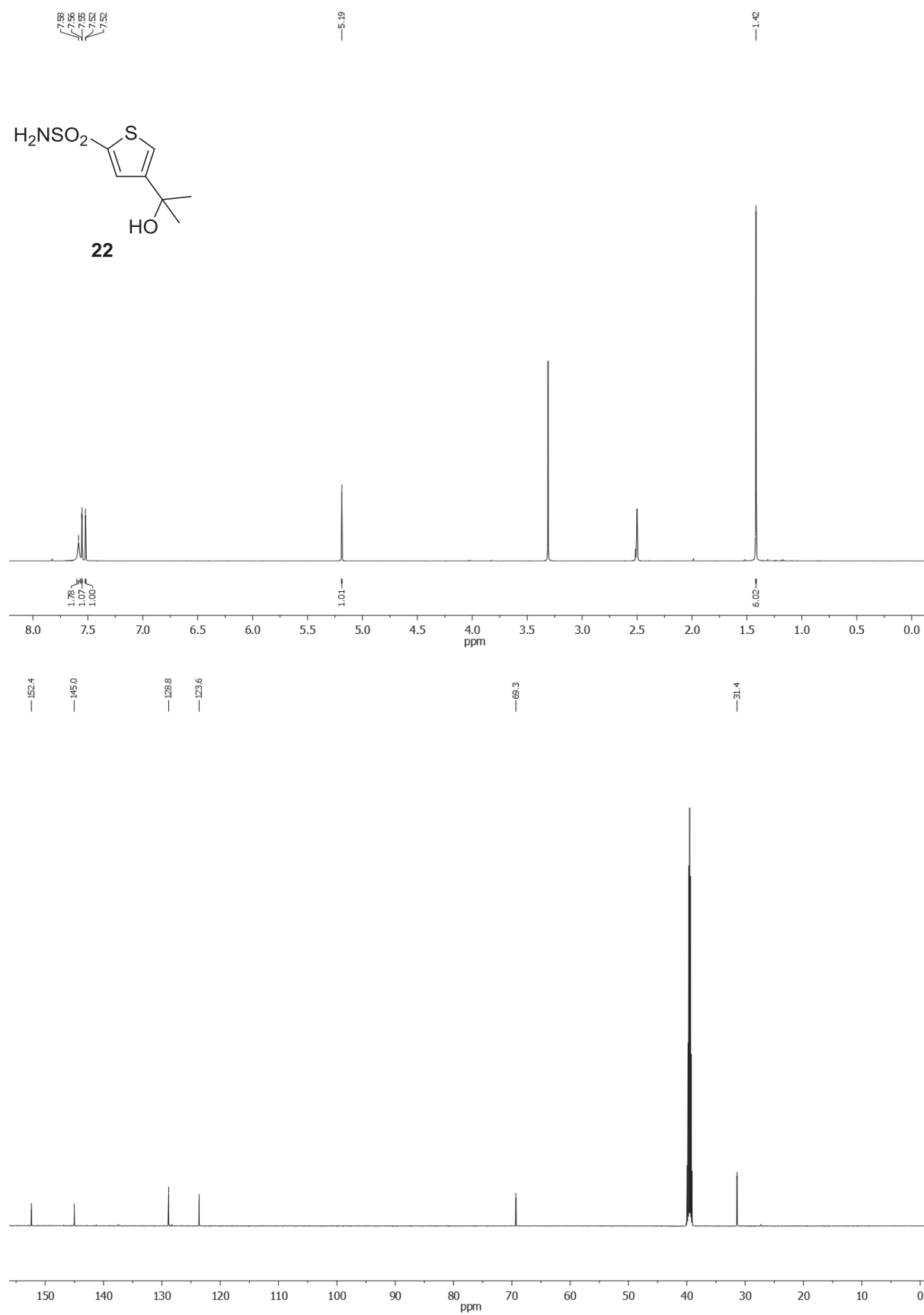
^1H and ^{13}C NMR spectra of compound **20**



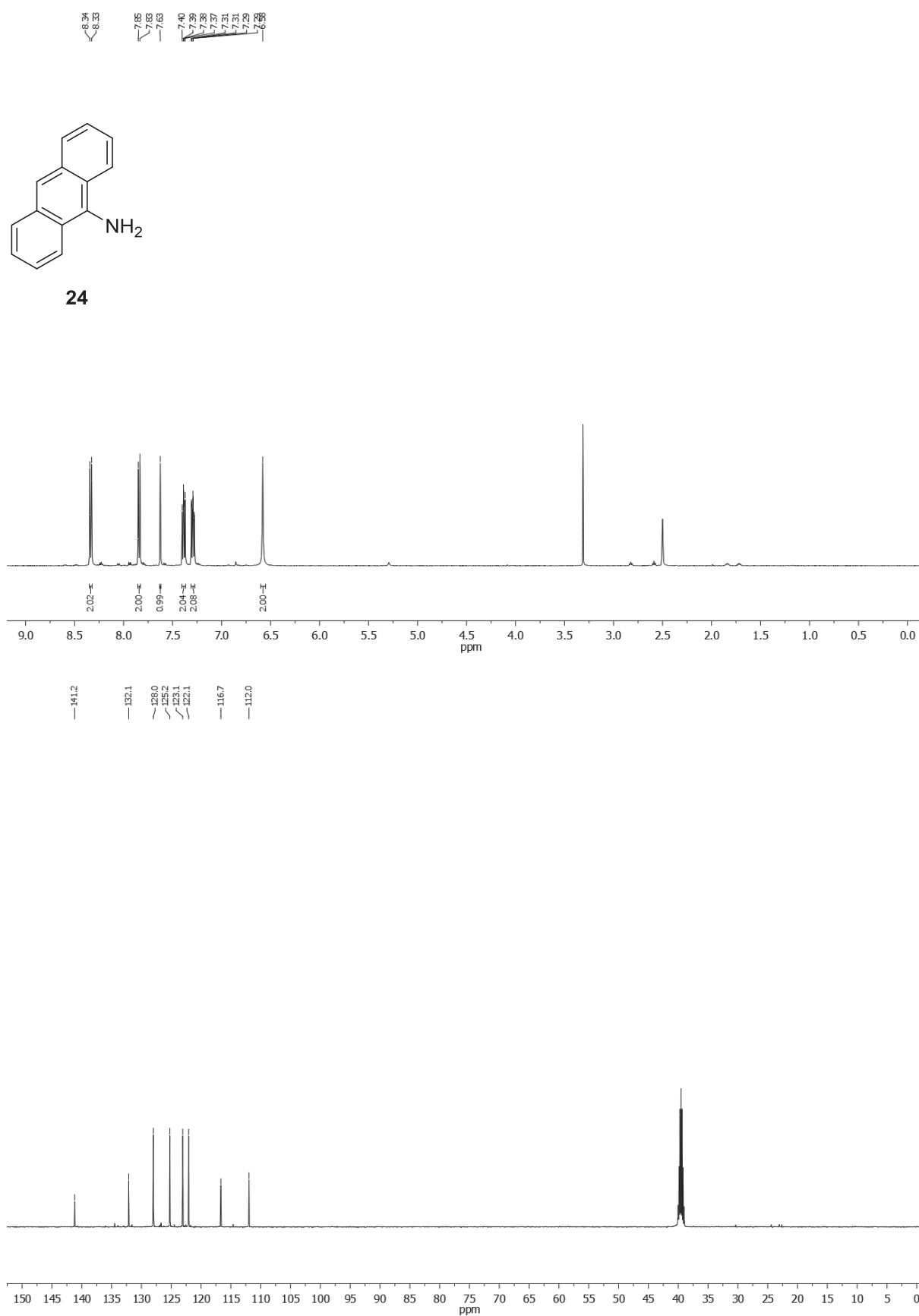
^1H and ^{13}C NMR spectra of compound **21**



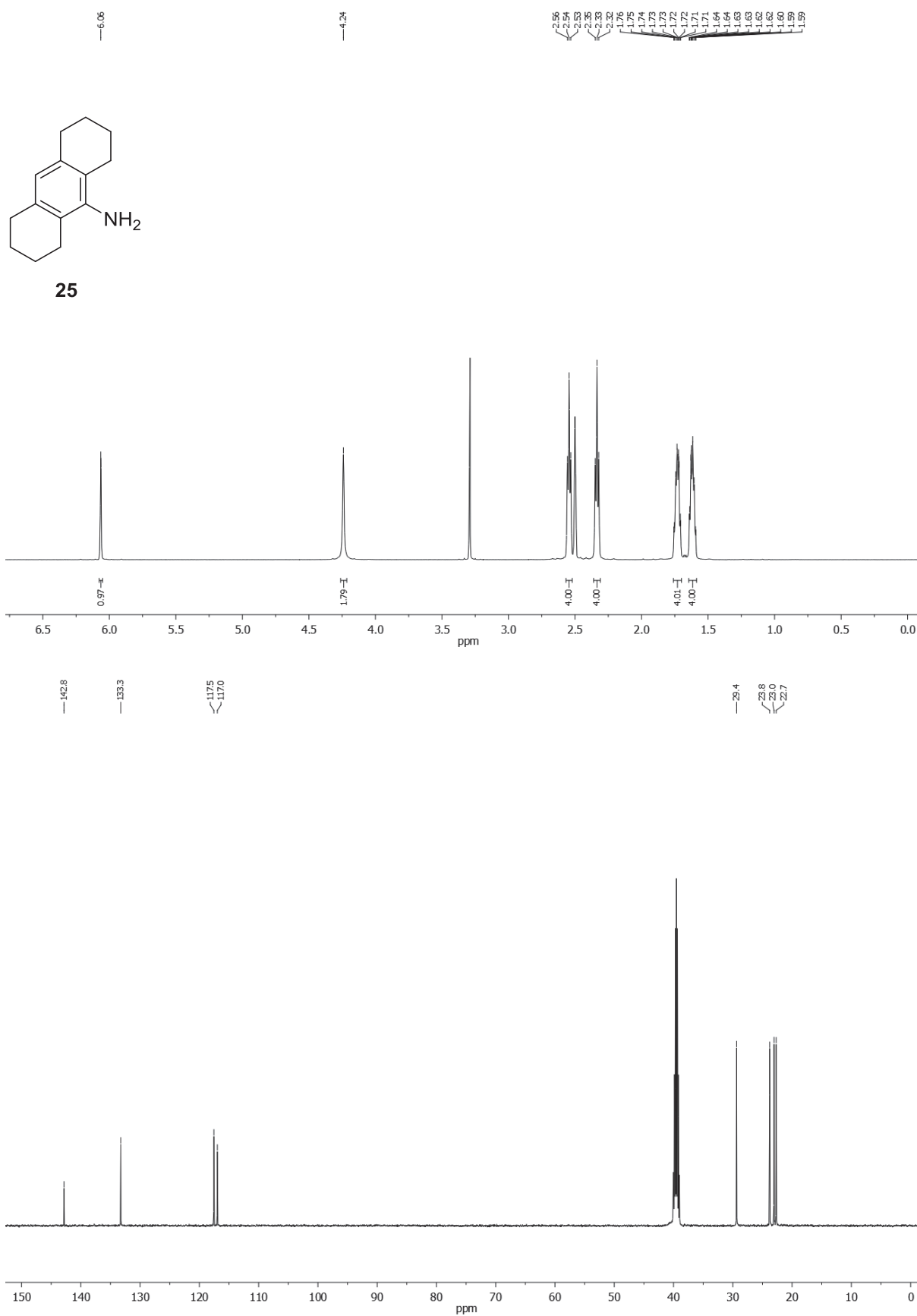
^1H and ^{13}C NMR spectra of compound **22**



^1H and ^{13}C NMR spectra of compound **24**



^1H and ^{13}C NMR spectra of compound **25**

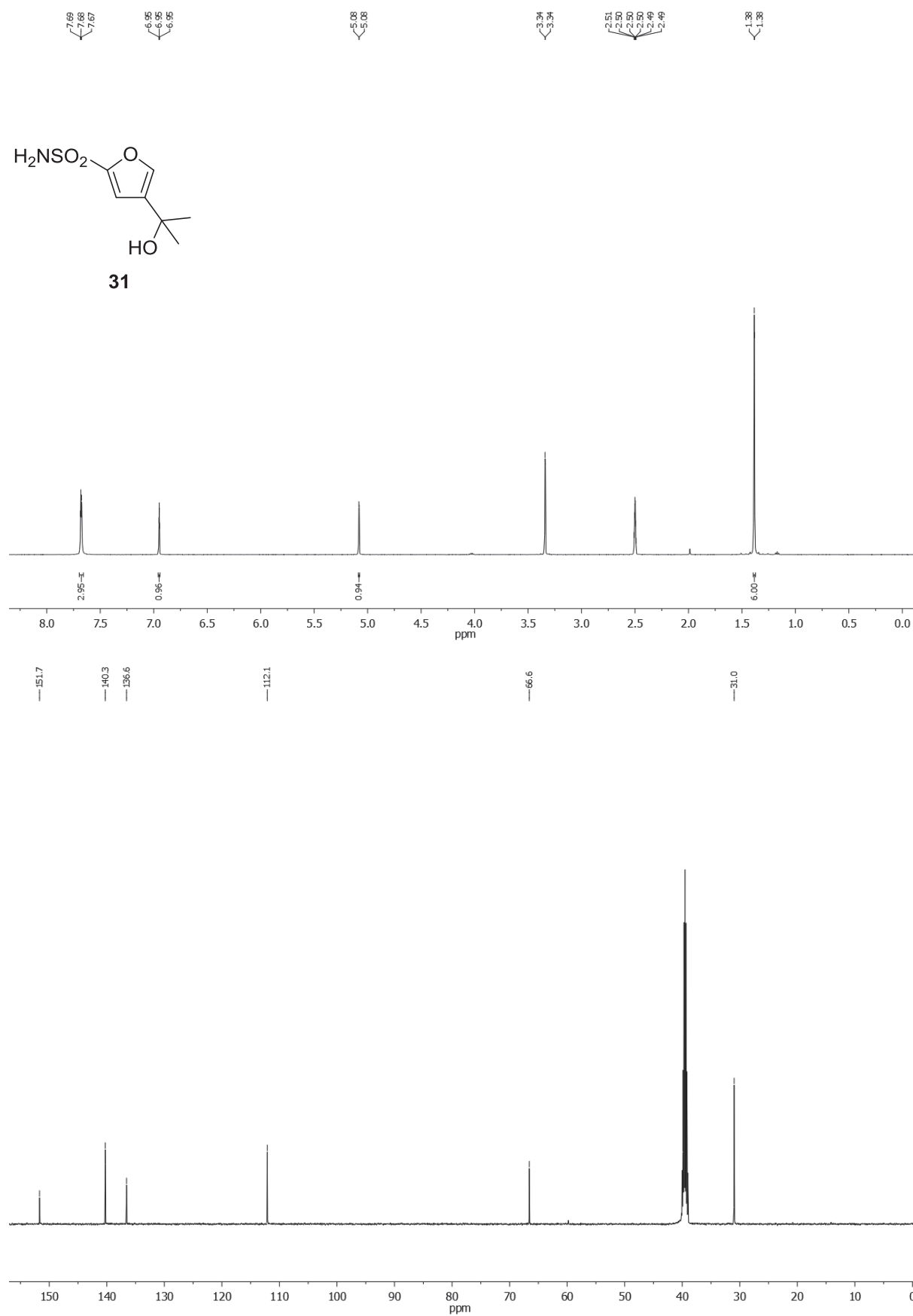


Chemical structure of compound **30** is shown above the spectra. The structure is 2-ethoxycarbonyl-5-sulfamoylthiophene.

¹H NMR spectrum (top): The spectrum shows peaks in the aromatic region (7.12–8.64 ppm) and aliphatic region (1.26–1.29 ppm). Integration values are provided below the peaks: 0.86, 2.00, 0.98, 2.19, and 3.00. The x-axis is labeled "ppm" and ranges from 9.5 to 0.0.

¹³C NMR spectrum (bottom): The spectrum shows peaks in the carbonyl region (150.4–161.3 ppm) and aliphatic region (14.1–60.8 ppm). The x-axis is labeled "ppm" and ranges from 160 to 0.

^1H and ^{13}C NMR spectra of compound **31**



HPLC Stability Measurements

HPLC measurements for compounds **1-6** and **8-12** were performed on a Merck-Hitachi LaChrom Elite instrument (Hitachi High-Tech Corporation, Tokyo, Japan) equipped with a L-2130 HPLC pump (with inline degasser), a L-2200 autosampler, a L-2300 column oven and a L-2455 diode array detector (DAD). Chromatograms were monitored at 205 nm. The HPLC was equipped with a RP-18 column (4 μ m, 125 $\times$ 4 mm, Nucleosil, Machery-Nagel, Düren, Germany). The column oven was adjusted to 37 °C. Chromatograms were obtained using the program EZChrom Elite (version 3.3.2, Agilent, Santa Clara, USA).

HPLC measurements for compound **7** were performed on a Jasco HPLC 2000 instrument (Jasco, Pfungstadt, Germany) equipped with a LG-2080-02S ternary low-pressure gradient unit, a DG-2080-53 degasser, a PU-2080 HPLC pump, a CO-2060 column oven and a UV-2075 UV detector. Chromatograms were monitored at 205 nm. The column oven was adjusted to 37 °C. Chromatograms were obtained using the program ChromPass (version 1.8.6.1, Jasco, Pfungstadt, Germany).

Chromatograms were recorded under isocratic conditions, using 60% H₂O_{dd} and 40% MeCN (v/v) (compound **1**), 55% H₂O_{dd} and 45% MeCN (v/v) (compounds **6**, **7**, **8**, **11**, **12**), 50% H₂O_{dd} and 50% MeCN (v/v) (compound **9**), 45% H₂O_{dd} and 55% MeCN (v/v) (compound **10**), respectively, the flow rate was 1.0 mL/min. Chromatograms of compound **4** were recorded using a linear gradient from 55% H₂O_{dd} and 45% MeCN (v/v) to 75% H₂O_{dd} and 25% MeCN, the flow rate was 1.2 mL/min. Chromatograms of compound **2** were recorded under isocratic conditions, using 63% H₂O_{dd} and 37% MeCN (v/v), the flow rate was 1.5 mL/min. Chromatograms of compound **3** and **5** were recorded under isocratic conditions, using 50% H₂O_{dd}, 30% MeCN (v/v) and 20% MeOH (v/v) (compound **3**) and 50% H₂O_{dd}, 35% MeCN (v/v) and 15% MeOH (v/v) (compound **5**), respectively, the flow rate was 1.5 mL/min. Each eluent (except for MeOH) contained 0.1% TFA.

Sample preparation and incubation for stability measurements

Five different buffers/solutions were prepared with H₂O_{dd} as follows and adusted with a Metrohm 691 pH Meter.

pH = 8.4, 20 mM Tris-HCl buffer, 150 mM NaCl

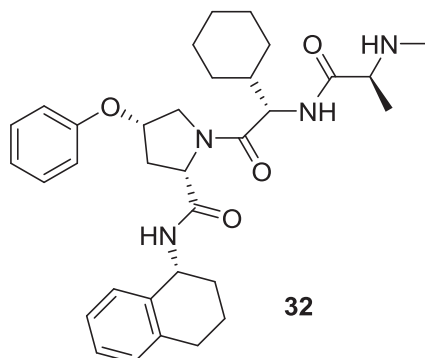
pH = 7.4, 12 mM phosphate buffer, 2,7 mM KCl, 137 mM NaCl

pH = 6.0, 12 mM phosphate buffer, 2,7 mM KCl, 137 mM NaCl

pH = 4.0, 10 mM NaOAc / AcOH

pH = 1.0, 100 mM HCl

Compound **32** served as an internal standard. The preparation is reported elsewhere.¹⁴



Stock solutions of all test compounds and of the internal standard compound were prepared in MeCN. For incubations, 400 μ L of the corresponding buffer and 100 μ L of the solutions of the respective test compound and of the internal standard compound were added. The final incubation volume was 500 μ L. The final concentrations of the test compound and the internal standard were 0.1 mM each. The concentration of MeCN was 20% (v/v). Incubations were performed at 37 °C using an Eppendorf Thermomixer comfort with 300 rpm. Aliquots of 20 μ L were injected into the HPLC system at the beginning of the incubations and in 60 min intervals over 8 h.

Areas under the curve (AUCs) of the resulting chromatograms were determined for the respective test compound and the internal standard. Ratios were calculated using the following equation 1,

$$\text{ratio} = \frac{\frac{\text{AUC}_{\text{compd}}(t)}{\text{AUC}_{\text{IS}}(t)} \times 100\%}{\frac{\text{AUC}_{\text{compd}}(t_0)}{\text{AUC}_{\text{IS}}(t_0)}} \quad (1)$$

where $\text{AUC}_{\text{compd}}(t)$ is the area under the curve of the compound at time t , and $\text{AUC}_{\text{IS}}(t)$ is the area under the curve of the internal standard at time t , $\text{AUC}_{\text{compd}}(t_0)$ is the area under the curve of the compound at time $t = 0$ h, and $\text{AUC}_{\text{IS}}(t_0)$ is the area under the curve of the internal standard at time $t = 0$ h.

Ratio values at nine different time points were plotted *versus* incubation time and fitted to the equation of exponential decay. From the obtained pseudo-first order rate constants, k , the half-lives, $t_{1/2}$, were calculated using equation 2.

$$t_{1/2} = \frac{\ln(2)}{k} \quad (2)$$

Sample preparation and incubation for decomposition analysis of sulfonylurea **2**

The chromatograms for the analysis of decomposition products of sulfonylurea **2** were recorded using a linear gradient (flow rate= 1.4 mL/min) starting with 80% H₂O_{dd} and 20% MeCN (v/v) to 55% H₂O_{dd} and 45% MeCN within 12.5 min and back to 80% H₂O_{dd} and 20% MeCN (v/v) within 10.5 min at 23.0 min. The eluents contained 0.1% TFA.

Stock solutions of the sulfonylurea **2** and the respective spiking compounds **17**, **22** and of the internal standard compound were prepared in MeCN. For incubations, 400 µL of 100 mM HCl (pH = 1.0) and 100 µL of the solutions of the respective compounds (either compound **2** alone, compound **2** together with compound **17** or compound **2** together with compound **22**) and of the internal standard compound were added. The final incubation volume was 500 µL. The final concentrations of the compounds and the internal standard were 0.1 mM each. Incubations were performed at 37 °C using an Eppendorf Thermomixer comfort with 300 rpm. Aliquots of 20 µL were injected into the HPLC at 0 h or 2 h of incubation.

Figures S1-S12. Time course of decomposition of compounds 1-12

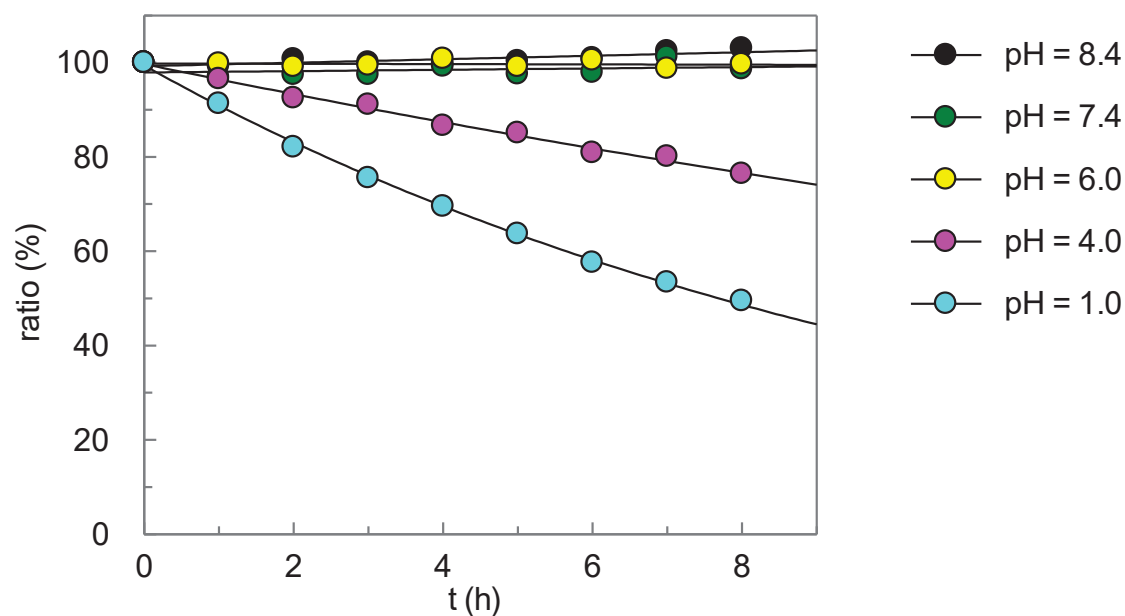


Figure S1. Time course of decomposition of MCC950 (**1**). Compound **1** (0.1 mM) was incubated at 37 °C for 8 h at five different pH values in the presence of an internal standard (0.1 mM) and subjected to HPLC analysis. The ratio is plotted *versus* incubation time.

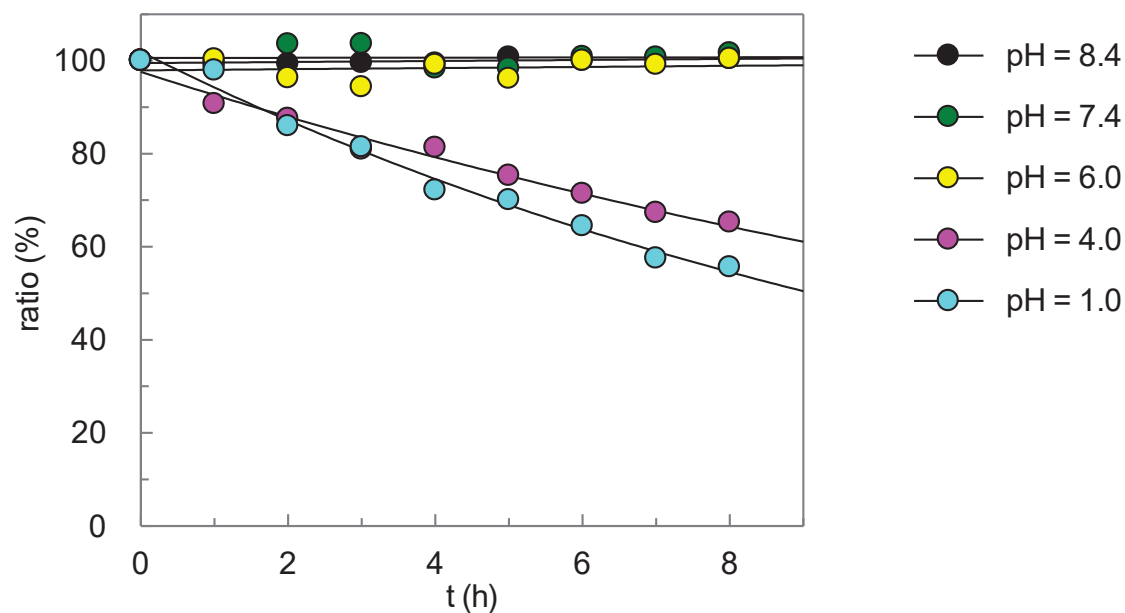


Figure S2. Time course of decomposition of sulfonylurea **2**. Compound **2** (0.1 mM) was incubated at 37 °C for 8 h at five different pH values in the presence of an internal standard (0.1 mM) and subjected to HPLC analysis. The ratio is plotted *versus* incubation time.

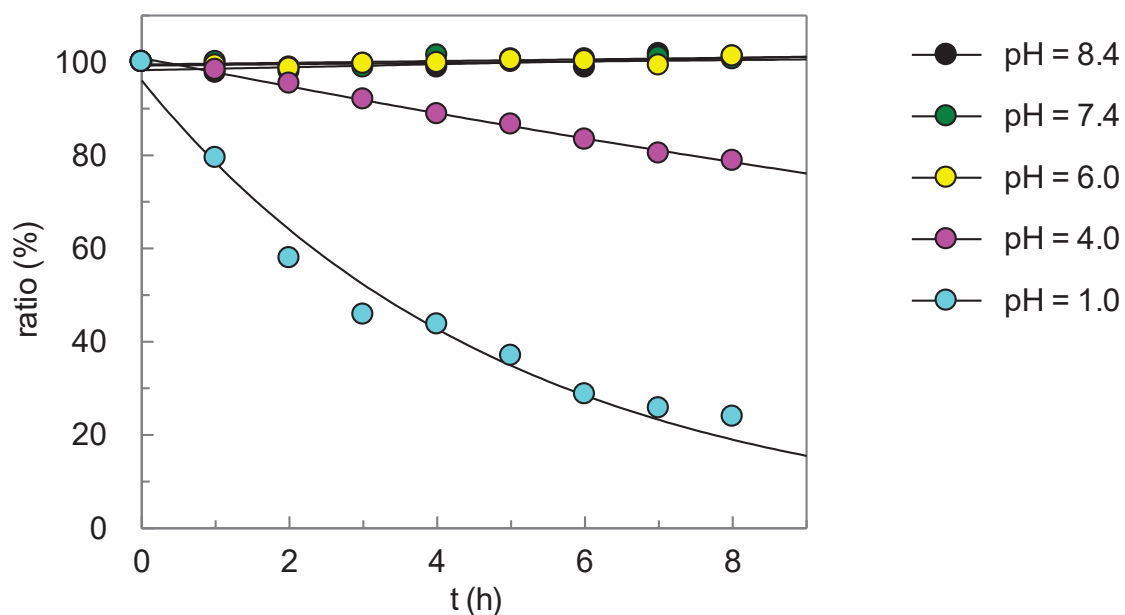


Figure S3. Time course of decomposition of sulfonylurea **3**. Compound **3** (0.1 mM) was incubated at 37 °C for 8 h at five different pH values in the presence of an internal standard (0.1 mM) and subjected to HPLC analysis. The ratio is plotted versus incubation time.

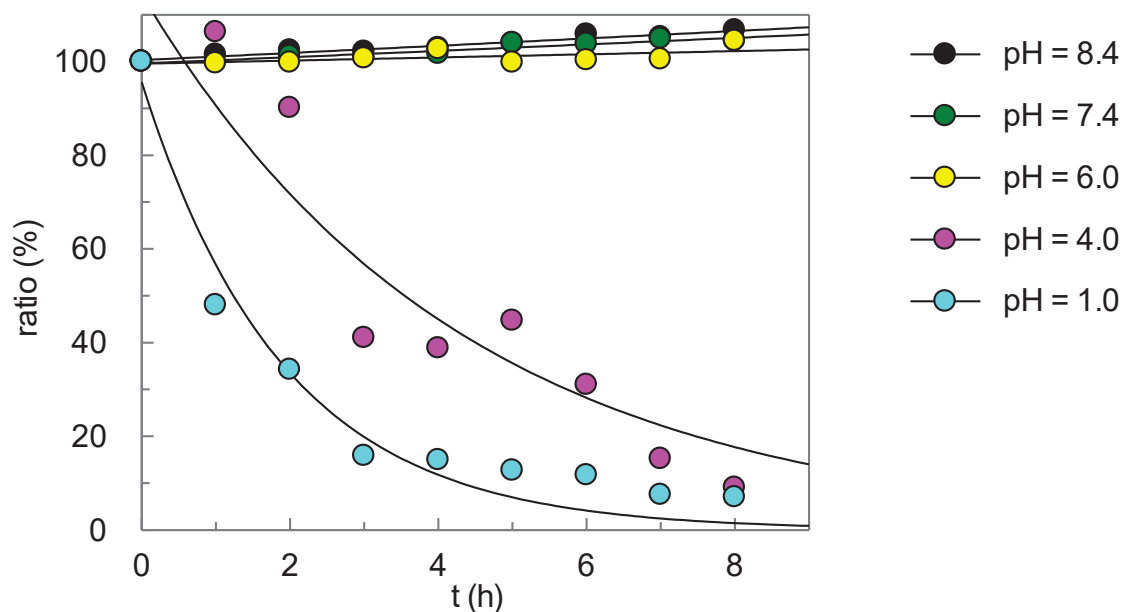


Figure S4. Time course of decomposition of sulfonylurea **4**. Compound **4** (0.1 mM) was incubated at 37 °C for 8 h at five different pH values in the presence of an internal standard (0.1 mM) and subjected to HPLC analysis. The ratio is plotted versus incubation time.

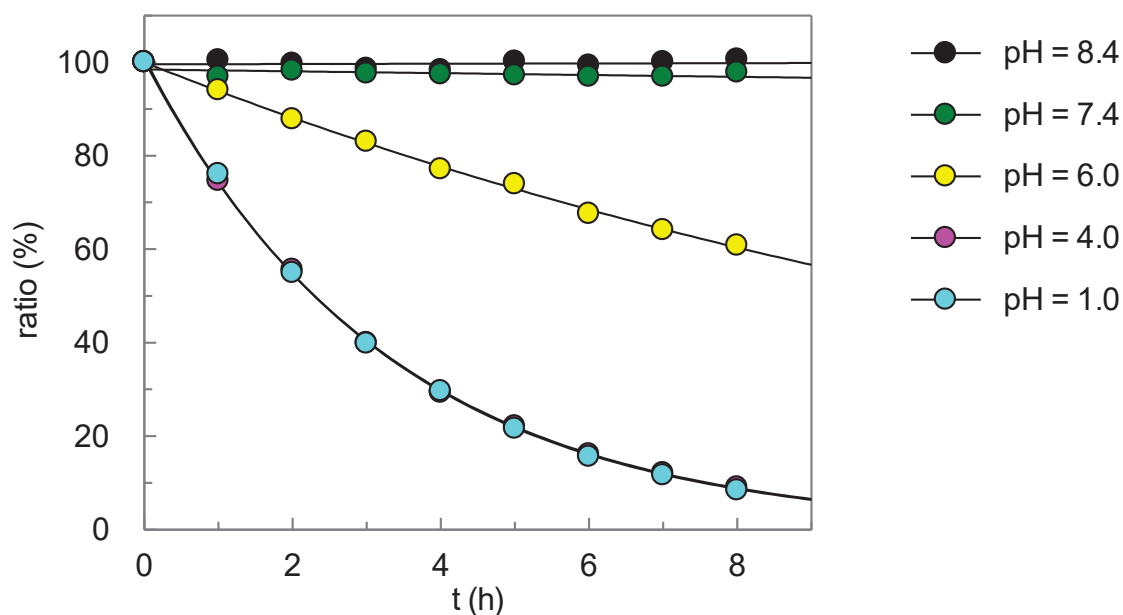


Figure S5. Time course of decomposition of sulfonylurea **5**. Compound **5** (0.1 mM) was incubated at 37 °C for 8 h at five different pH values in the presence of an internal standard (0.1 mM) and subjected to HPLC analysis. The ratio is plotted versus incubation time.

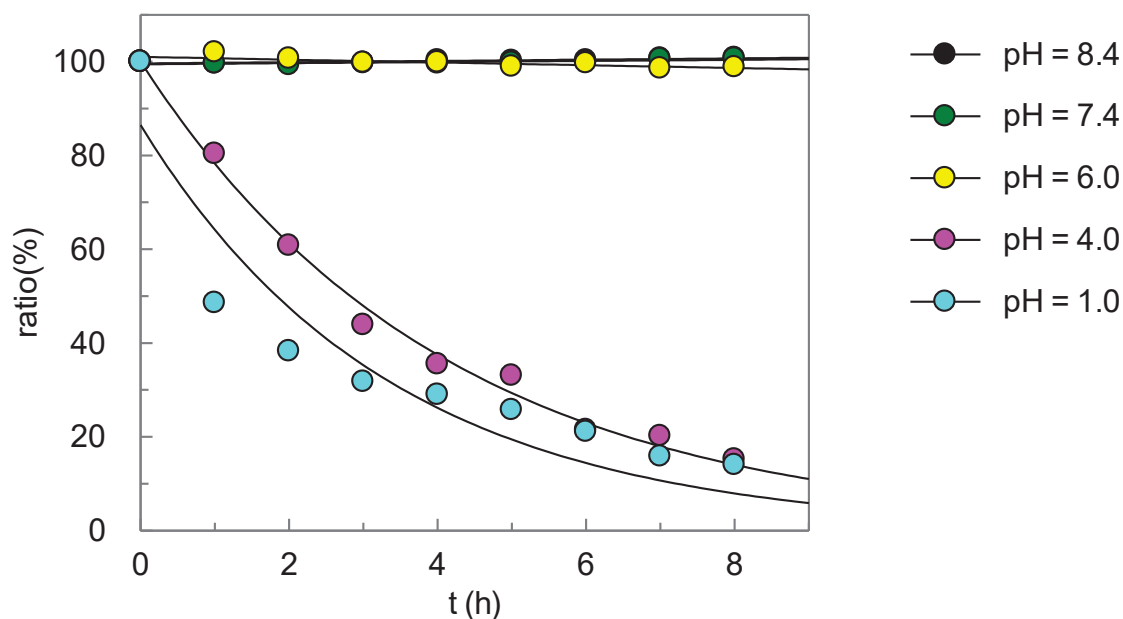


Figure S6. Time course of decomposition of sulfonylurea **6**. Compound **6** (0.1 mM) was incubated at 37 °C for 8 h at five different pH values in the presence of an internal standard (0.1 mM) and subjected to HPLC analysis. The ratio is plotted versus incubation time.

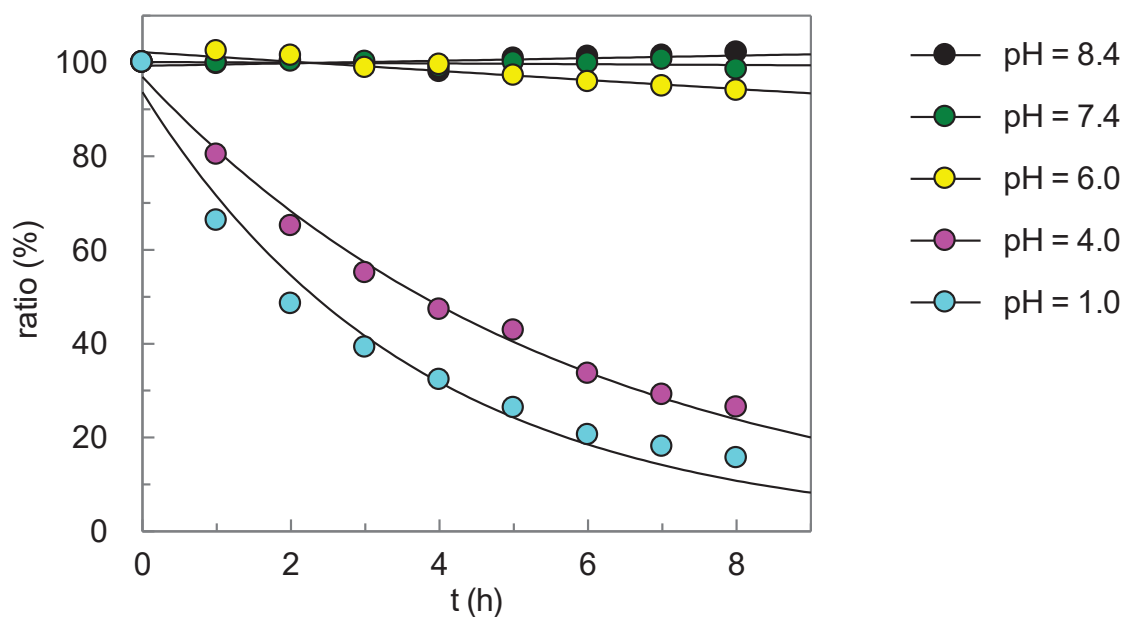


Figure S7. Time course of decomposition of sulfonylurea **7**. Compound **7** (0.1 mM) was incubated at 37 °C for 8 h at five different pH values in the presence of an internal standard (0.1 mM) and subjected to HPLC analysis. The ratio is plotted versus incubation time.

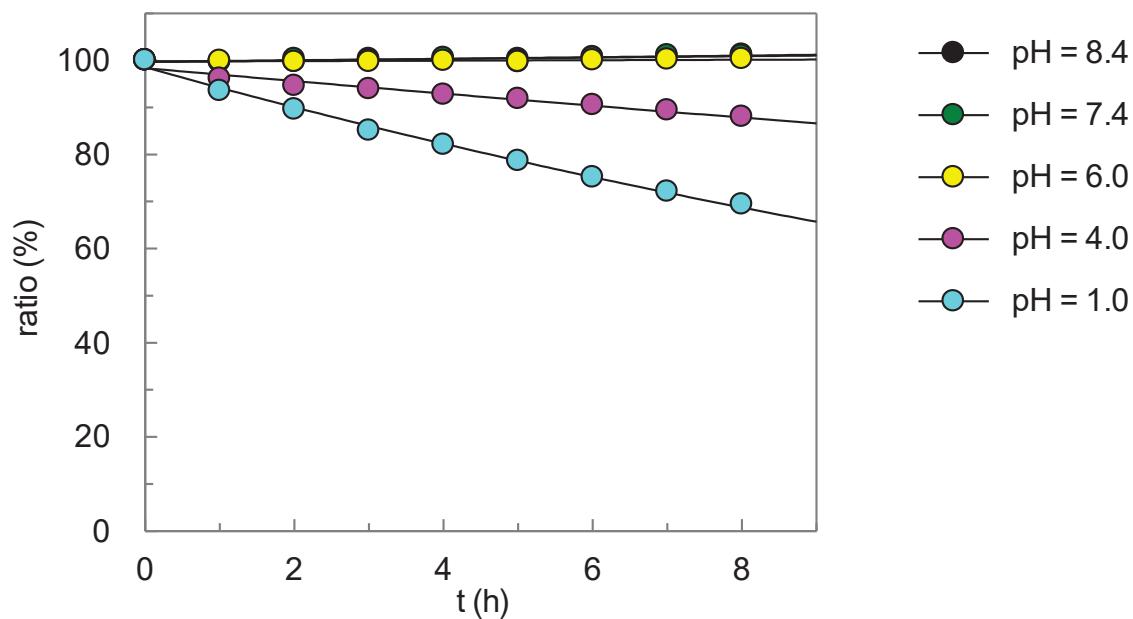


Figure S8. Time course of decomposition of sulfonylurea **8**. Compound **8** (0.1 mM) was incubated at 37 °C for 8 h at five different pH values in the presence of an internal standard (0.1 mM) and subjected to HPLC analysis. The ratio is plotted versus incubation time.

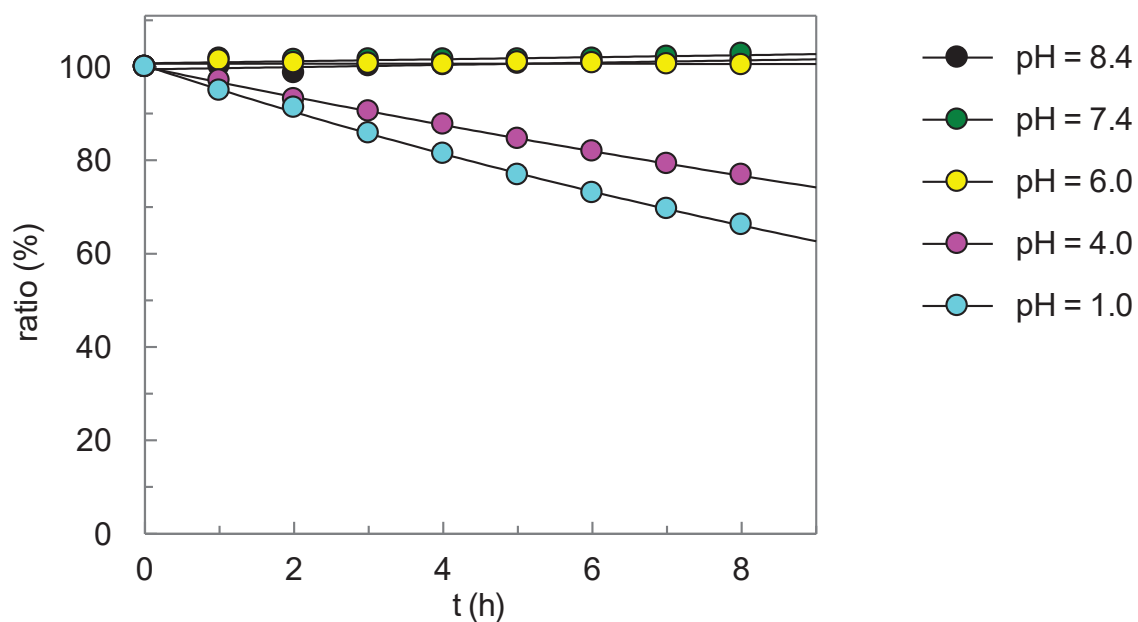


Figure S9. Time course of decomposition of sulfonylurea **9**. Compound **9** (0.1 mM) was incubated at 37 °C for 8 h at five different pH values in the presence of an internal standard (0.1 mM) and subjected to HPLC analysis. The ratio is plotted versus incubation time.

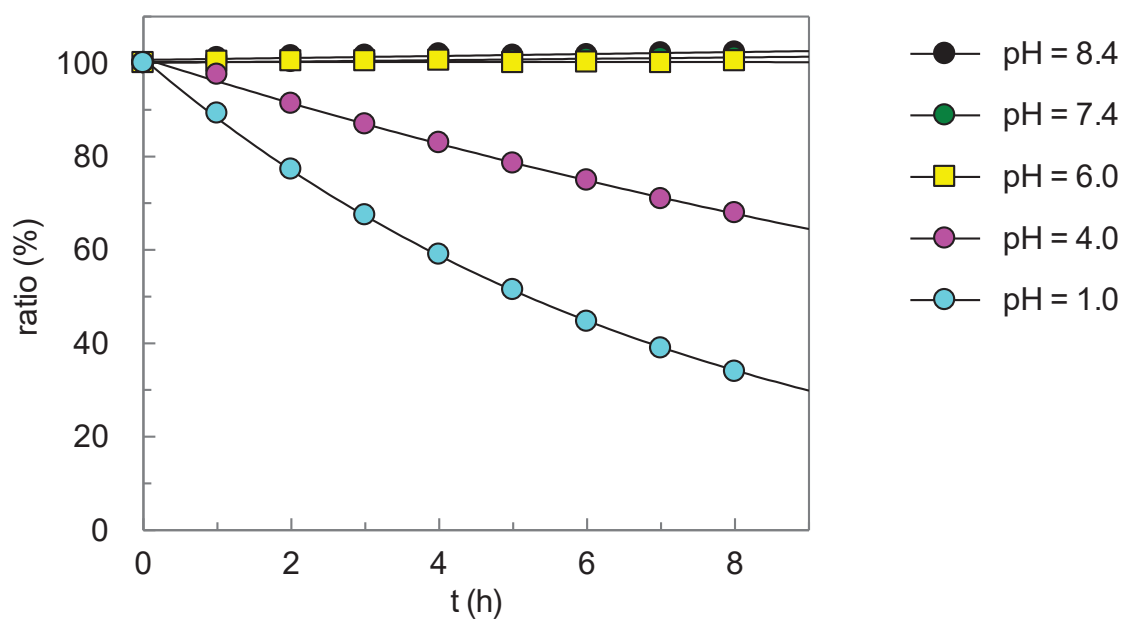


Figure S10. Time course of decomposition of sulfonylurea **10**. Compound **10** (0.1 mM) was incubated at 37 °C for 8 h at five different pH values in the presence of an internal standard (0.1 mM) and subjected to HPLC analysis. The ratio is plotted versus incubation time.

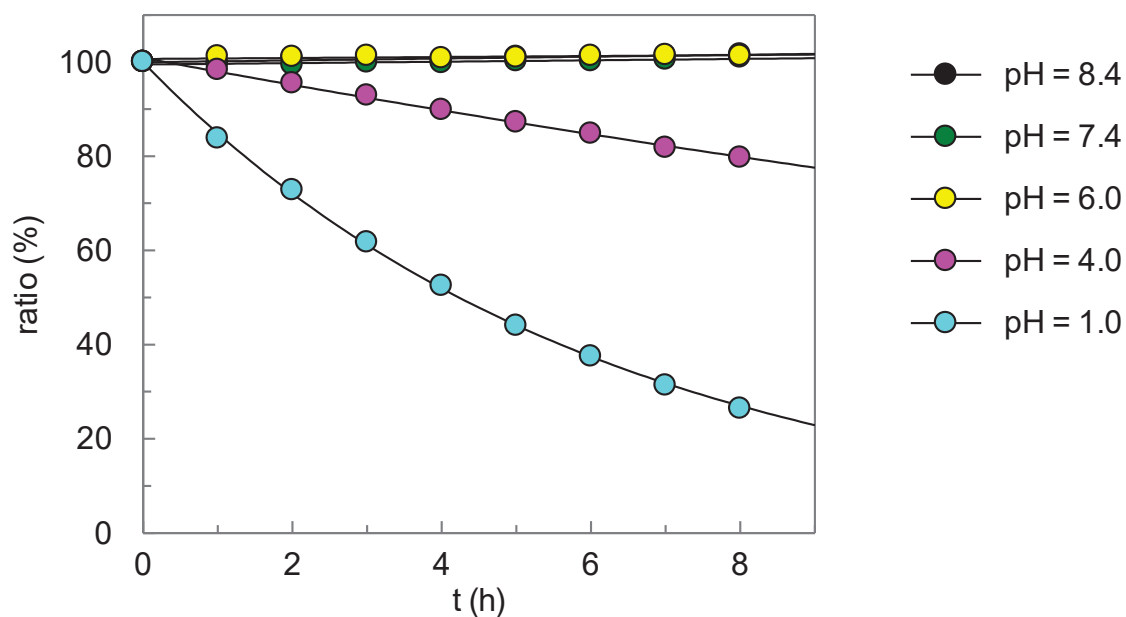


Figure S11. Time course of decomposition of sulfonylurea **11**. Compound **11** (0.1 mM) was incubated at 37 °C for 8 h at five different pH values in the presence of an internal standard (0.1 mM) and subjected to HPLC analysis. The ratio is plotted versus incubation time.

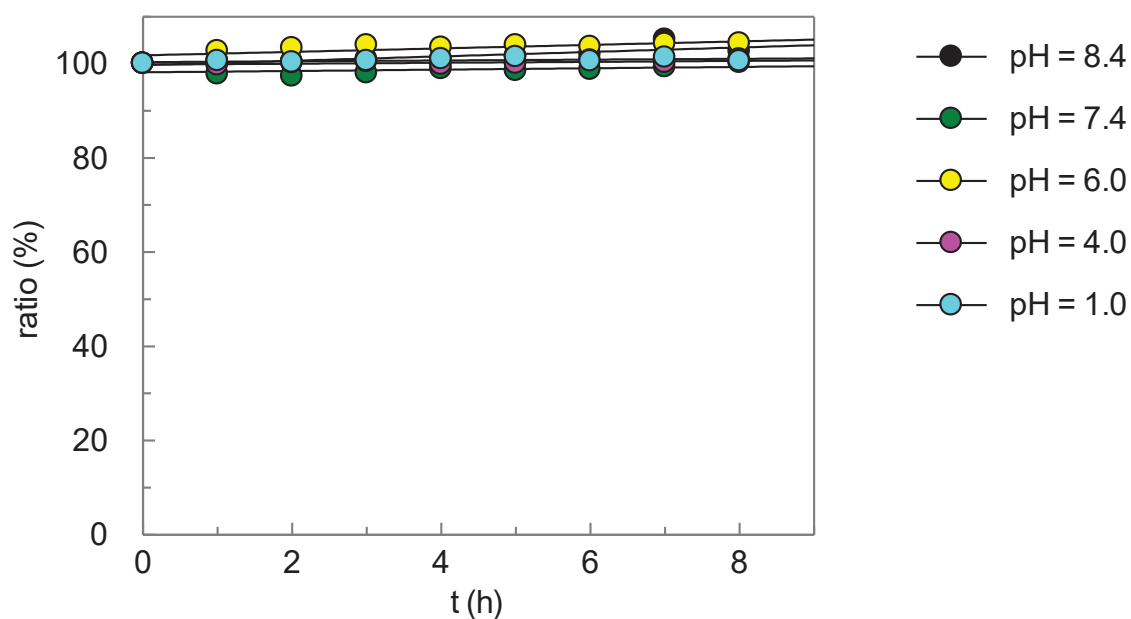
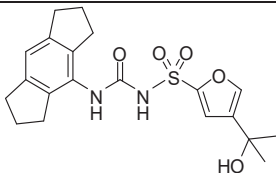
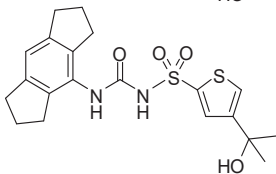
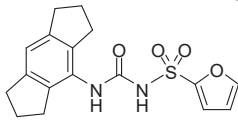
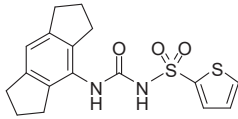
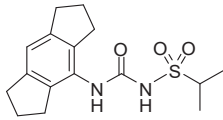
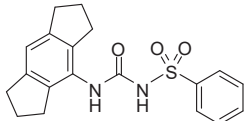
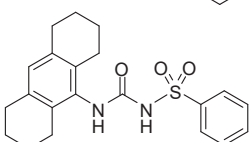
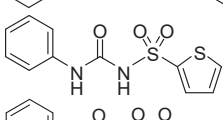
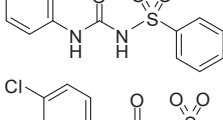
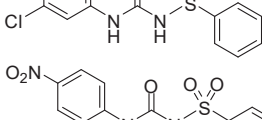
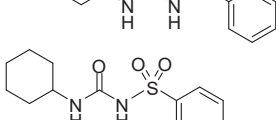
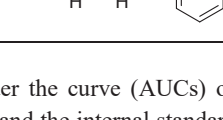


Figure S12. Time course of decomposition of sulfonylurea **12**. Compound **12** (0.1 mM) was incubated at 37 °C for 8 h at five different pH values in the presence of an internal standard (0.1 mM) and subjected to HPLC analysis. The ratio is plotted versus incubation time.

Table S1. Sulfonylureas prepared and their chemical stability

compd		ratio of compound concentration after 8 h (%) ^a half-life (h) ^b				
		pH = 1.0	pH = 4.0	pH = 6.0	pH = 7.4	pH = 8.4
1		ratio = 50% $t_{1/2}$ = 7.8 h	ratio = 76% $t_{1/2}$ = 21.1 h	ratio = 100% $t_{1/2}$ = n.d.	ratio = 99% $t_{1/2}$ = n.d.	ratio = 103% $t_{1/2}$ = n.d.
2		ratio = 56% $t_{1/2}$ = 8.9 h	ratio = 65% $t_{1/2}$ = 13.3 h	ratio = 100% $t_{1/2}$ = n.d.	ratio = 102% $t_{1/2}$ = n.d.	ratio = 100% $t_{1/2}$ = n.d.
3		ratio = 24% $t_{1/2}$ = 3.4 h	ratio = 79% $t_{1/2}$ = 22.1 h	ratio = 101% $t_{1/2}$ = n.d.	ratio = 101% $t_{1/2}$ = n.d.	ratio = 101% $t_{1/2}$ = n.d.
4		ratio = 7% $t_{1/2}$ = 1.3 h	ratio = 9% $t_{1/2}$ = 3.0 h	ratio = 104% $t_{1/2}$ = n.d.	ratio = 104% $t_{1/2}$ = n.d.	ratio = 107% $t_{1/2}$ = n.d.
5		ratio = 8% $t_{1/2}$ = 2.3 h	ratio = 9% $t_{1/2}$ = 2.3 h	ratio = 61% $t_{1/2}$ = 11.0 h	ratio = 98% $t_{1/2}$ = n.d.	ratio = 101% $t_{1/2}$ = n.d.
6		ratio = 14% $t_{1/2}$ = 2.3 h	ratio = 15% $t_{1/2}$ = 2.8 h	ratio = 99% $t_{1/2}$ = n.d.	ratio = 101% $t_{1/2}$ = n.d.	ratio = 101% $t_{1/2}$ = n.d.
7		ratio = 16% $t_{1/2}$ = 2.6 h	ratio = 26% $t_{1/2}$ = 4.0 h	ratio = 94% $t_{1/2}$ = n.d.	ratio = 99% $t_{1/2}$ = n.d.	ratio = 102% $t_{1/2}$ = n.d.
8		ratio = 69% $t_{1/2}$ = 15.4 h	ratio = 88% $t_{1/2}$ = 49.2 h	ratio = 100% $t_{1/2}$ = n.d.	ratio = 101% $t_{1/2}$ = n.d.	ratio = 101% $t_{1/2}$ = n.d.
9		ratio = 66% $t_{1/2}$ = 13.3 h	ratio = 77% $t_{1/2}$ = 20.9 h	ratio = 100% $t_{1/2}$ = n.d.	ratio = 103% $t_{1/2}$ = n.d.	ratio = 101% $t_{1/2}$ = n.d.
10		ratio = 34% $t_{1/2}$ = 5.1 h	ratio = 68% $t_{1/2}$ = 13.9 h	ratio = 100% $t_{1/2}$ = n.d.	ratio = 101% $t_{1/2}$ = n.d.	ratio = 102% $t_{1/2}$ = n.d.
11		ratio = 26% $t_{1/2}$ = 4.2 h	ratio = 80% $t_{1/2}$ = 23.7 h	ratio = 101% $t_{1/2}$ = n.d.	ratio = 101% $t_{1/2}$ = n.d.	ratio = 102% $t_{1/2}$ = n.d.
12		ratio = 101% $t_{1/2}$ = n.d.	ratio = 101% $t_{1/2}$ = n.d.	ratio = 104% $t_{1/2}$ = n.d.	ratio = 100% $t_{1/2}$ = n.d.	ratio = 103% $t_{1/2}$ = n.d.

^aAreas under the curve (AUCs) of the chromatograms after 8 h incubation at the indicated pH were determined for the test compound and the internal standard. Ratios were calculated as a measure of compound stability using eq 1. ^bRatio values at nine different time points were plotted *versus* incubation time and fitted to the equation of exponential decay. Half-lives, $t_{1/2}$, were calculated using eq 2. ^cNot detectable; no decomposition observed within 8 h.

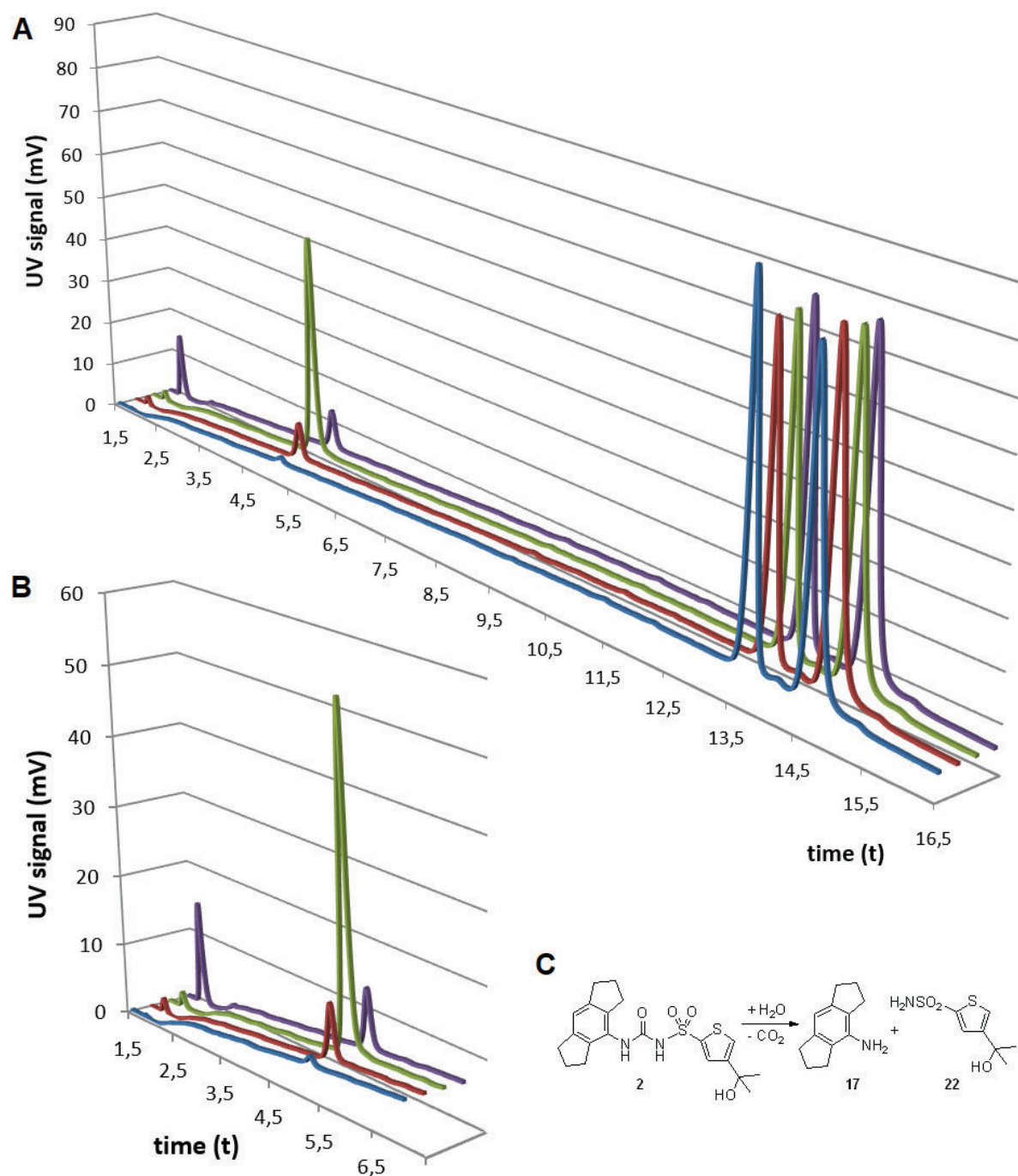


Figure S13. (A) Chromatograms for decomposition analysis of sulfonylurea **2** from 1.5 min to 16.5 min and (B) sections from 1.5 min to 7.0 min. Compound **2** eluted at 13.8 min, 1,2,3,5,6,7-hexahydro-*s*-indacen-4-amine (**17**) at 5.3 min, 4-(2-hydroxypropan-2-yl)thiophene-2-sulfonamide (**22**) at 1.8 min and the internal standard **32** at 14.7 min. Blue: Compound **2** after 0 h of incubation. Red: Compound **2** after 2 h of incubation. Green: Compound **2** spiked with **17** after 2 h of incubation. Purple: Compound **2** spiked with **22** after 2 h of incubation. (C) Decomposition reaction of sulfonylurea **2**.

Surface Plasmon Resonance Spectroscopy

Surface plasmon resonance spectroscopy (SPR) experiments were performed at 25 °C on a Biacore 8K device (GE Healthcare) that was equipped with a streptavidin functionalized sensor chip (Series S Sensor Chip SA, Cytiva). The system was flushed with running buffer (10 mM HEPES pH 7.4, 200 mM NaCl, 0.5 mM ADP, 0.5 mM dithiothreitol (DTT), 2 mM MgCl₂, 1 g/L carboxymethyl dextran (CMD), 0.05% Tween20, 2% DMSO) before the sensor chip was conditioned with three consecutive injections of 1 M NaCl in 50 mM NaOH (1 min, 10 µL/min). Biotinylated NLRP3-NACHT protein was recombinantly expressed in HEK-293T cells and after purification immobilized onto the sensor chip at 2 µL/min for 3000 s. Residual streptavidin binding sites were blocked by for consecutive injections of Biotin-PEG (1 µM, M_n 2,300 Da) for 2 min at 10 µL/min. Binding of compounds was measured in single cycle mode by injecting increasing concentrations of 2.3 to 600 nM at 30 µL/min (association 240 s, dissociation 60/360 s). Data were collected at a rate of 10 Hz, double referenced by blank cycle and reference flow cell subtraction and corrected by a 4-point solvent correction. For determination of binding constants, processed data were fitted to a 1:1 interaction model using the Biacore Insight Evaluation Software (version 3.0.12.15655).

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Appendix III. Publication III: Development of Fluorescent and Biotin Probes Targeting NLRP3

The following pages include the article “Development of Fluorescent and Biotin Probes Targeting NLRP3” as it was published in Frontiers in Chemistry by Frontiers Media S.A..

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Development of Fluorescent and Biotin Probes Targeting NLRP3

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Extracellular signals drive the nucleation of the NLRP3 inflammasome which leads to the release of cytokines and causes inflammatory events. Hence, the inflammasome has gained enormous momentum in biomedical basic research. The detailed mechanisms of inflammasome generation and regulation remain to be elucidated. Our study was directed toward the design, convergent synthesis, and initial biochemical evaluation of activity-based probes addressing NLRP3. For this purpose, probes were assembled from a CRID3/MCC950-related NLRP3-binding unit, a linker portion and a coumarin 343 fluorophore or biotin. The affinity of our probes to NLRP3 was demonstrated through SPR measurements and their cellular activity was confirmed by reduction of the interleukin 1 β release from stimulated bone marrow-derived macrophages. The initial characterizations of NLRP3-targeting probes highlighted the coumarin probe **2** as a suitable tool compound for the cellular and biochemical analysis of the NLRP3 inflammasome.

Keywords: NLRP3, probes, surface plasmon resonance, inflammasome, CRID3, MCC950

INTRODUCTION

NLRP3 (NOD-, LRR- and PYD-containing protein 3) has attracted increasing attention as an important player in the (patho)physiology of inflammations. NLRP3 (or NALP3 or cryopyrin) is a tripartite protein of the family of nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs). It contains an N-terminal pyrin domain (PYD), a C-terminal leucine-rich repeat (LRR) domain, and a central NACHT domain. The NACHT domain exists in NAIP (neuronal apoptosis inhibitory protein), CIITA (MHC class II transcription activator), HET-E (incompatibility locus protein from *Podospora anserina*), and TP1 (telomerase-associated protein) proteins and consists itself of four subdomains, i.e., of a nucleotide-binding domain, the helical domain 1, a winged-helix domain, and the helical domain 2 (Eldeeb et al., 2019; Vande Walle et al., 2019). The NLRP3 inflammasome represents a key mediator of the inflammatory response. Inflammasomes are intracellular supramolecular complexes. Their formation is triggered either by damage-associated (such as ATP) or pathogen-associated (such as nigericin) molecular patterns or by Toll-like receptor activation (Baldwin et al., 2016; Chauhan et al., 2020). NLRP3 activation is controlled by PYD and NACHT domain phosphorylation (Song et al., 2017; Stutz et al., 2017). In the NLRP3 inflammasome, NLRP3 acts as a sensor and forms a platform together with the adaptor protein ASC (apoptosis-associated speck-like protein containing a CARD) and the effector protein caspase-1. Crucial steps for NLRP3 inflammasome assembly are NLRP3 oligomerization and recruitment of ASC to NLRP3 oligomers. NLRP3 operates through caspase-1 activation which, in turn, results in the processing of cytokine pro-forms and the release of the matured pro-inflammatory interleukins IL-1 β and IL-18. Caspase 1-catalyzed cleavage of gasdermin D (GSDMD) generates

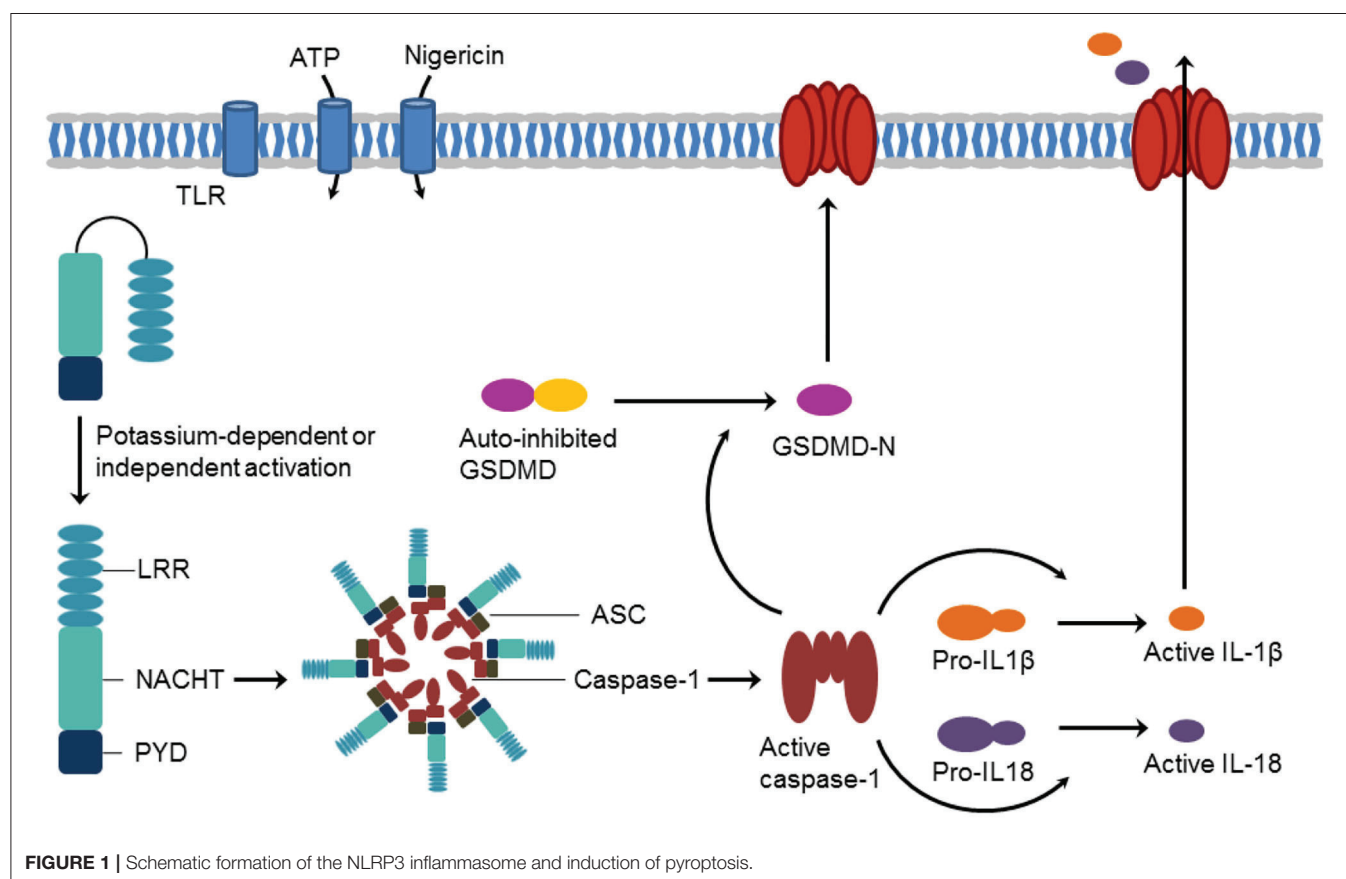
a pore-forming N-terminal protein (GSDMD-N) initiates a lytic form of cell death, referred to as pyroptosis (**Figure 1**) (Dubois et al., 2019; Pfalzgraff and Weindl, 2019; Broz et al., 2020).

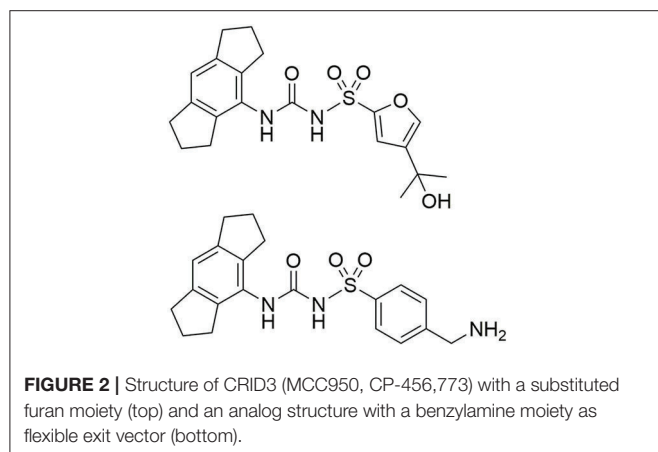
NLRP3 is associated with a variety of disorders such as central nervous system (CNS) diseases, rheumatoid arthritis, gout, atherosclerosis, asthma, and crystal nephropathy (Ludwig-Portugall et al., 2016; Dhana et al., 2018; Mangan et al., 2018; Chauhan et al., 2020). The cryopyrin-associated periodic syndromes (CAPS) are autoinflammatory disorders caused by various gain-of-function missense mutations of the NLRP3 gene (Mangan et al., 2018). The strong inflammatory role of NLRP3 provides the impetus for the development of drugs, acting as “NLRP3 inflammasome blockers” (Cocco et al., 2014). On the one hand, agonists could be useful to reverse the immunosuppressive conditions in tumors. On the other hand, inhibitors of the NLRP3 pathway are promising candidates for the treatment of several chronic and auto-inflammatory diseases, including those for which adequate therapies currently do not exist (Mangan et al., 2018). Accordingly, NLRP3 was long considered as a target for the development of small molecule inhibitors. A broad spectrum of NLRP3 inhibitors has been found, from natural products to small synthetic molecules. Cytokine-release-inhibitory-drug-3

(CRID3), a diarylsulfonylurea derivative, showed potent inhibition of the NLRP3 inflammasome formation (Coll et al., 2015). This compound CRID3 is also known as MCC950 or CP-456,773 (**Figure 2**). Due to reversible binding to the NACHT domain of wildtype NLRP3, ASC oligomerization could be blocked resulting in decreased IL-1 β release. It was reported that CRID3/MCC950 binds in proximity to the Walker B motif in NLRP3, impeding the ATP hydrolysis and leading to an inactive NLRP3 conformation (Coll et al., 2019; Tapia-Abellán et al., 2019). Nevertheless, further research attempts are required to decipher NLRP3 inflammasome formation and activation. In this study, we developed new fluorescent and biotin-tagged activity-based probes which could be achieved through a convergent synthetic strategy. These probes are intended to be used, e.g., for fast competition assays, pulldown experiments or confocal microscopy.

RESULTS AND DISCUSSION

Covalent and non-covalent conjugation of proteins to fluorophores is commonly used to study their cellular localization, to discover protein-protein interactions, and





to label proteins in their native environment. Fluorescence can be provided by means of a fluorescent low-molecular weight probe possessing sufficient affinity for the target protein. A widely used class of fluorophores, 7-aminocoumarins, are characterized by chemical and enzymatic stability, a small molecular size and large Stokes shifts (Breidenbach et al., 2020). Rigidization of the amino group due to cyclization resulted in increased quantum yields and restored fluorescence in aqueous media. A prominent example is coumarin 343 with an emission maximum at 480 nm in aqueous media after excitation at 440 nm, which is frequently employed to assemble activity-based probes (Terai and Nagano, 2008; Meimetis et al., 2014; Mertens et al., 2014; Kohl et al., 2015). The appendage of a biotin moiety to a bioactive probe offers the opportunity to elucidate and analyze intracellular binding partners of the probe. Biotinylation profits from the exceedingly strong interaction between biotin and either avidin or streptavidin (Trippier, 2013; Verdoes and Verhelst, 2016; Chakrabarty et al., 2019). Accordingly, in order to provide tool compounds to unravel hitherto unknown processes of inflammasome activation and dynamics, we designed NLRP3-specific probes with either coumarin 343 or biotin.

CRID3/MCC950 (Figure 2), the best-known and prevalently applied NLRP3 inflammasome inhibitor, constitutes a sulfonylurea with a hexahydro-s-indacen-4-yl group at the terminal nitrogen and a furan moiety at the sulfur atom (Vande Walle et al., 2019; Chauhan et al., 2020; Wu et al., 2020). ¹¹C-labeled CRID3/MCC950 was developed for non-invasive PET imaging studies (Hill et al., 2020a). CRID3/MCC950 was chosen as the template for the NLRP3 binding moiety for all of our probes. First, we had to identify an appropriate exit vector to connect either a fluorescent label or a biotin moiety to the NLRP3 ligand without losing entire binding affinity to the target. Recently published structure-activity relationships concerning diarylsulfonylurea-based NLRP3 inhibitors revealed the intact western indacene moiety of CRID3/MCC950 to be crucial for biological activity, and hence for target binding. On the other hand, structural alterations on the eastern sulfonamide part were better tolerated (Hill et al., 2017, 2020b; Agarwal et al., 2020).

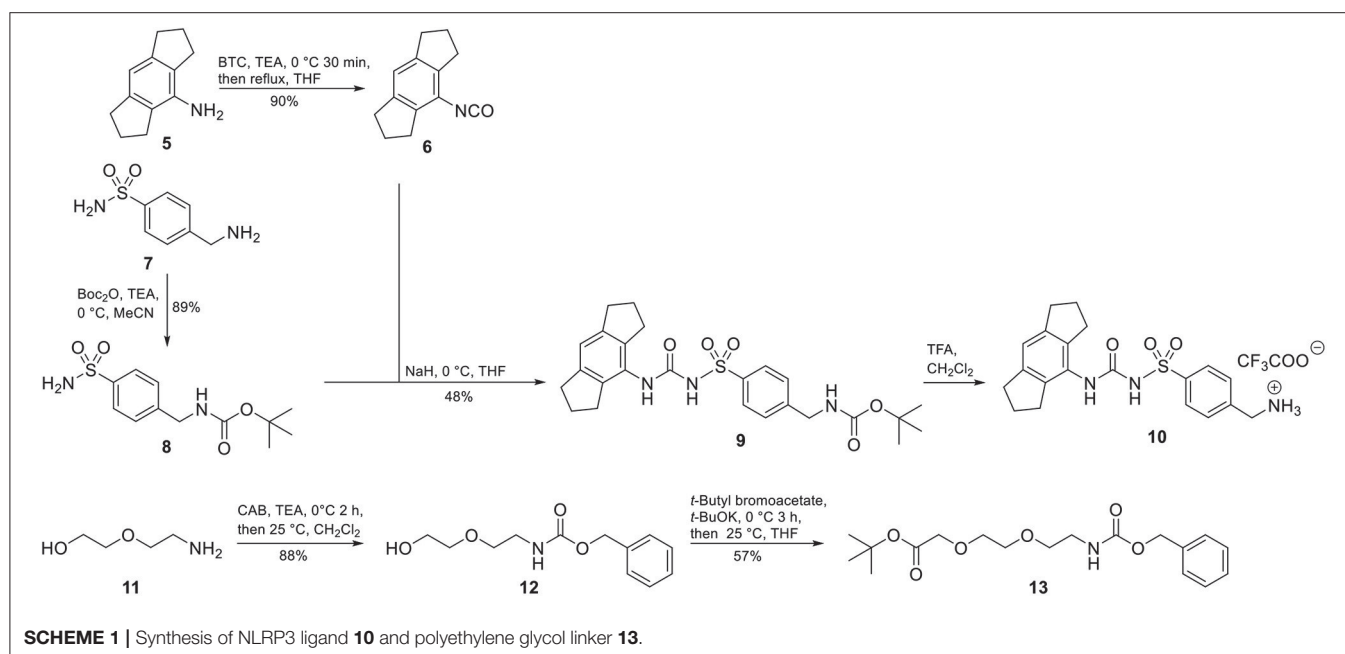
Two recently developed CRID3/MCC950-derived photoaffinity probes bearing a benzophenone photophore followed the same structural design (Coll et al., 2019; Vande Walle et al., 2019). Similarly, a trifluoromethyl phenyl diazirine photo-cross-linker was introduced at the western part of CRID3/MCC950 whose indacene unit was equipped with a bromine mass tag (Hill et al., 2020b). These photoaffinity probes were employed to covalently label NLRP3 (Coll et al., 2019; Vande Walle et al., 2019).

Accordingly, our molecular design toward NLRP3 probes was based on the introduction of a molecular handle for attaching the label at the eastern part of the diarylsulfonylurea. A network of convergent synthetic routes was employed to assemble the envisaged probes. As depicted in Scheme 1, we synthesized an aromatic sulfonamide (8) containing a Boc-protected amino group to be used as exit vector at a later stage of the synthesis. Next, we transformed the hexahydro-s-indacen-4-amine 5 in the presence of triphosgene (BTC, [bis(trichloromethyl) carbonate]) into the isocyanate 6. Following the pretreatment of sulfonamide 8 with sodium hydride, the deprotonated intermediate was reacted with isocyanate 6 to obtain the NLRP3 ligand 9. Compound 9 was deprotected to the trifluoroacetic acid (TFA) salt 10 prior to each coupling reaction.

For two final compounds, a short polyethylene glycol linker was chosen to connect the NLRP3 binding moiety and coumarin 343 or biotin. 2-(2-Aminoethoxy)ethanol (11) was Cbz-protected and the resulting alcohol 12 was O-alkylated with *tert*-butyl bromoacetate to obtain the orthogonally protected linker 13 (Scheme 1), which, in turn, was N-deprotected under palladium on carbon (Pd/C)-catalyzed conditions to 14 (Scheme 2).

A Knoevenagel condensation of isopropylidene malonate (15) with 8-hydroxyjulolidine-9-carboxaldehyde (16) delivered coumarin 343 (17) as fluorescent moiety (Scheme 2). HATU-promoted coupling of 17 with the deprotected linker 14 led to compound 18. After deprotection of 18, it was coupled with the NLRP3 binding moiety 10 to obtain the fluorescent NLRP3 probe 1. For a shorter coumarin-labeled probe, coumarin 343 (17) was directly connected to the NLRP3 ligand 10 to achieve probe 2. To obtain the biotin-tagged probes, we first activated D-biotin with *N*-hydroxysuccinimide (NHS) to the active ester 20. This was reacted with the deprotected linker 14. After deprotection of 21, a further HATU coupling was performed to yield the biotin-tagged probe 3. Direct linkage of 20 with the NLRP3 ligand 10 delivered the short biotin-tagged probe 4.

Surface plasmon resonance (SPR) is a powerful and versatile spectroscopic method, but less commonly used for the analysis of low-molecular weight probes for functional proteins. SPR spectroscopy allows a real-time measurement and the label-free detection of biomolecular interactions through a surface-sensitive response. In order to assess the suitability of our probes, they were subjected to an SPR analysis and compounds 1–4, together with CRID3/MCC950, were analyzed for their binding behavior to human NLRP3. For that purpose, recombinant biotinylated human NLRP3-PYD-NACHT domain protein derived from HEK293T cells was immobilized on a streptavidin-functionalized sensor chip (sensor chip SA). After a stabilization period, the analytes, CRID3/MCC950 and the respective test

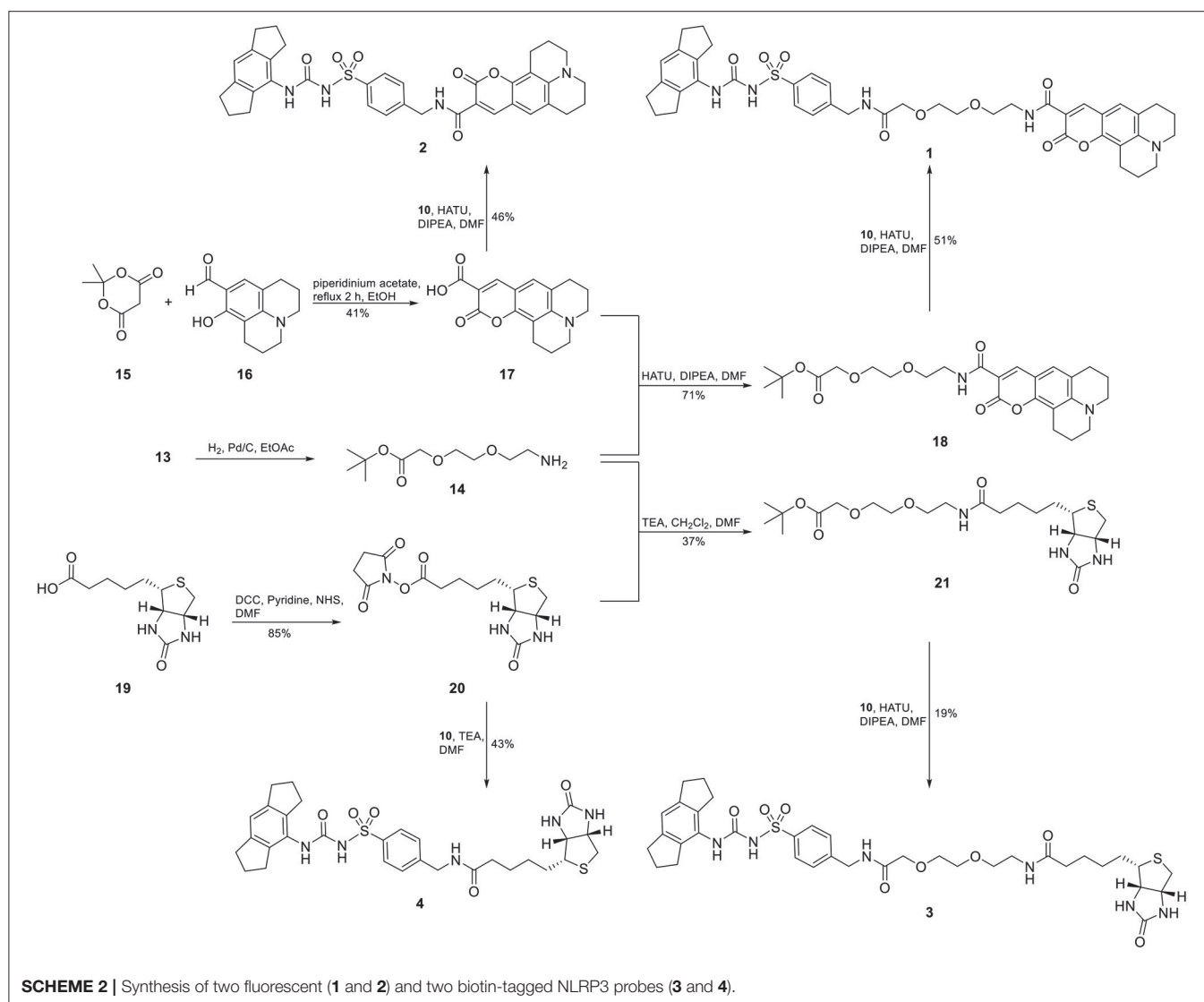


compound, were injected on single channels using single-cycle kinetics (**Figure 3** for compounds **2** and **4**; for compounds **1** and **3**, see **Supplementary Figure 1**). The SPR data (**Table 1**) revealed a dissociation constant K_D of 322 nM for the fluorescently labeled compound **2** and a K_D value of 31 nM for CRID3/MCC950. The dissociation constant K_D for the fluorescently labeled probe with the PEG linker (**1**) was determined to be 813 nM, whereas the two biotinylated probes (**3**, **4**) bound with K_D values of 1,030 and 611 nM, respectively. These data confirm that the molecular probes **1–4** attached with fluorescent or biotin labels to the scaffold of CRID3/MCC950 bind to recombinant NLRP3 protein. The smaller compounds (**2** and **4**) exhibited lower dissociation constants than their linker-connected counterparts (**1** and **3**). To confirm that compound **2** and **4** have the same binding site as CRID3/MCC950, we injected two ligands 120 s after the first injection (**Figure 3D**). No significant response change, indicative of a second binding site, was observed in either succession of the injections (left and right), which points to the assumption that CRID3/MCC950 and both compounds interact with the same binding site on the target protein NLRP3. Although CRID3/MCC950 possessed a higher affinity to NLRP3, our compounds bound sufficiently tight, making them promising candidates for a further characterization as suitable NLRP3 probes. However, the reversible binding mode may obstruct the use for Western blotting analysis.

Next, the probes were examined in cell culture experiments. First, we determined the cytotoxicity of probes **1–4** at different concentrations between 625 nM and 40 μ M in immortalized murine bone marrow-derived macrophages (**Figure 4**). While biotin probes **3** and **4** were proved to be non-toxic up to 40 μ M, and the coumarin probe **1** was non-toxic up to 10 μ M, the coumarin probe **2** exhibited some cytotoxicity at concentrations higher than 2.5 μ M.

The following experiments were performed with probes **1–4** in immortalized murine bone marrow-derived macrophages. Nigericin, an antibiotic carboxylate ionophore, was used as the activator of the NLRP3 inflammasome, owing to its ability to induce a potassium efflux. In addition, we addressed the absent in melanoma 2 (AIM2) inflammasome, a distinct inflammasome complex, which, upon assembly, can also activate caspase-1 and cause cells to release IL-1 β (Hornung et al., 2009). For this purpose, AIM2 was stimulated by transfection with poly(deoxyadenylic-deoxythymidylic) acid [poly(dA:dT)] after pretreatment with LPS. A Homogeneous Time Resolved Fluorescence (HTRF) assay was employed to determine the release of IL-1 β (**Figure 5**). Probe **1** was used at 2.5 μ M and 10 μ M, i.e., with concentrations found to be non-toxic in the same cells. We observed a complete inhibition of IL-1 β release at a concentration of 10 μ M, but not at 2.5 μ M. Probe **2** was only investigated at 2.5 μ M due to toxic effects at higher concentration. Notably, this probe showed complete inhibition at 2.5 μ M, just like CRID3/MCC950 at this concentration. The biotin-tagged probes **3** and **4**, at 20 μ M each, did not significantly affect the cytokine release (see **Supplementary Figure 2**). For reasons to be clarified, these cellular data only partially agree with the affinity of the entire subset of compounds **1–4** for NLRP3 as determined in the SPR assay. The coumarin-containing compound **2** at 20 μ M also reduced AIM2-dependent IL-1 β release initiated by poly(dA:dT) (data not shown), obviously due to cytotoxicity. However, at 2.5 μ M, only a slightly reduced AIM2-dependent release of IL-1 β was observed (**Figure 5**). As expected, CRID3/MCC950 and probe **1** did not affect this process.

To assess pyroptosis, cytosolic lactate dehydrogenase (LDH) release was measured as a marker of cell lysis. In contrast to priming with lipopolysaccharide (LPS) alone,



priming with LPS followed by treatment with nigericin and poly(dA:dT), respectively, clearly increased cell lysis. CRID3/MCC950 and probe **2**, each at a concentration of 2.5 μ M, as well as probe **1** at 10 μ M prevented the NLRP3-dependent, but not the AIM2-dependent cell death (**Figure 5**). This rescue effect can be attributed to the anti-pyrototic activity of the compounds and was in agreement with the inhibitory potency of the investigated compounds regarding IL-1 β release.

The most suitable probe, **2**, was subjected to confocal microscopy for detection of NLRP3 in non-stimulated, immortalized murine macrophages overexpressing NLRP3 fused to mCitrine, a monomeric variant of the yellow fluorescent protein. The confocal images show that NLRP3 staining was colocalized with the yellow fluorescence (**Figure 6A**). On the other hand, the presence of the NLRP3 fusion protein was a prerequisite for colocalization as shown in the negative control (**Figure 6B**). This incipient result indicated

a successful application of this coumarin-labeled NLRP3 ligand for future studies.

In conclusion, we designed and synthesized four probes based on the structure of the prototypical NLRP3 inhibitor CRID3/MCC950. An appropriate exit vector was established for the attachment of a fluorescent or biotin label. All of our probes were demonstrated to bind to the NLRP3 protein. Two coumarin-labeled probes were shown to reach the cytosolic target and to block the final cellular response. One of them, probe **2**, is expected to serve as an appropriate tool compound in the field of inflammasome research which is currently ongoing in our laboratories.

EXPERIMENTAL SECTION

General Experimental Procedures

Chemicals were purchased from ABCR, BLDpharm, Fisher Chemical, Sigma Aldrich and Tokyo Chemical Industry. Thin

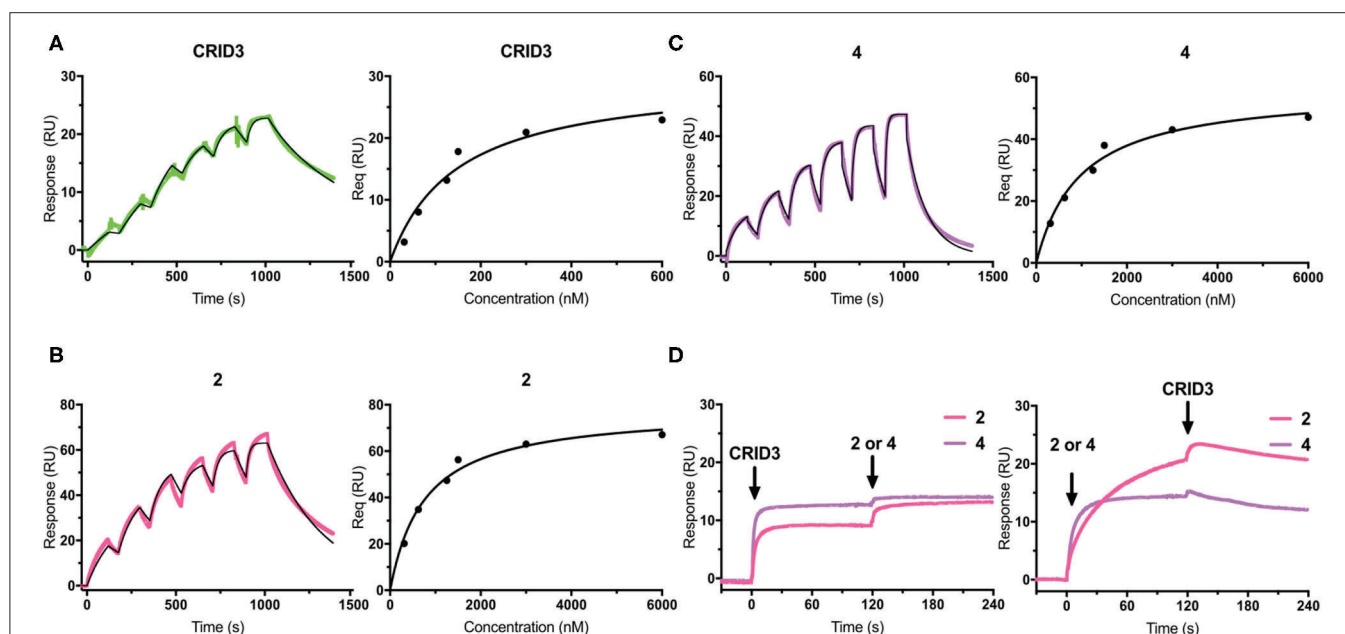


FIGURE 3 | (A–C) SPR sensorgrams (left) and concentration-binding response unit curves (right) of CRID3/MCC950 (**A**), compound **2** (**B**), and compound **4** (**C**) injected on the chip surface loaded with human NLRP3-PYD-NACHT. The binding parameters obtained from the sensorgrams are listed in **Table 1**. Data were fitted to a 1:1 binding model. **(D)** SPR sensorgrams of CRID3/MCC950, compound **2**, and compound **4** injected in competition mode on the chip surface loaded with human NLRP3-PYD-NACHT. Either CRID3/MCC950 (left) or compound **2** or **4** (right) were provided in the first injection step, followed by a second injection step after 120 s, where equimolar concentrations of the second compound were injected in presence of the first compound.

TABLE 1 | Second-order on-rate constants for association (k_a), first-order off-rate constants for dissociation (k_d), and kinetic dissociation constants (K_D) for NLRP3 ligands.

Compound	k_a ($M^{-1}s^{-1}$)	k_d (s^{-1})	K_D (nM)
CRID3/MCC950	7.5×10^4	2.3×10^{-3}	31
1	1.3×10^4	1.0×10^{-2}	813
2	1.0×10^4	3.3×10^{-3}	322
3	2.2×10^4	2.2×10^{-2}	1,030
4	1.4×10^4	8.8×10^{-3}	611

layer chromatography was carried out with pre-coated silica gel (60 F254) aluminum sheets from Merck. Detection was performed with UV light at 254 and 360 nm or with $AgNO_3$ staining. Acros Organics silica gel 60 (70–230 mesh) was taken for preparative column chromatography. Preparative silica gel flash column chromatography was performed on an Interchim Puriflash PF420 system with diode-array detection (DAD) from 200 to 400 nm. Melting points were measured on a Büchi 510 oil bath apparatus. HR-ESI-MS were recorded on a Bruker micrOTOF-Q mass spectrometer coupled with a HPLC Dionex UltiMate 3000 or a LTQ Orbitrap XL. ESI-MS mass spectra were recorded on an API2000 mass spectrometer coupled with an Agilent HPLC HP1100 using an EC50/2 Nucleodur C18 Gravity 3 μm column. The purity of synthesized compounds was determined by HPLC-DAD obtained on an Agilent HP1100 LC-MS instrument and the purity of all final

compounds was confirmed to be >95% (HPLC-DAD). NMR spectra were recorded on a Bruker Avance DRX 500 (500 MHz ^1H -NMR, 126 MHz ^{13}C -NMR) and a Bruker Avance III 600 (600 MHz ^1H -NMR, 151 MHz ^{13}C -NMR). Chemical shifts are given in parts per million (ppm) referring to the signal center using the solvent peaks for reference: DMSO- d_6 (2.49/39.7).

Syntheses

4-Isocyanato-1,2,3,5,6,7-hexahydro-s-indacene (**6**)

Triphosgene (BTC; 0.53 g, 1.8 mmol) was dissolved in dry THF (30 mL) at 0 °C under nitrogen atmosphere. The solution was stirred for 30 min at 0 °C. Triethylamine (TEA; 0.36 g, 3.6 mmol) was added in one portion and a solution of 1,2,3,5,6,7-hexahydro-s-indacene-4-amine (**5**, 0.31 g, 1.8 mmol) in dry THF (30 mL) was added dropwise over 30 min. The reaction mixture was refluxed for 30 min. All volatiles were evaporated. The residue was taken up in dry THF (30 mL), filtered and concentrated to yield a crude brown oil, which was directly used in the next step without further purification. Yield 90%; R_f = 0.67 (hexane).

tert-Butyl (4-Sulfamoylbenzyl)carbamate (**8**)

4-Aminomethylbenzenesulfonamide $\times$ HCl (**7**, 2.23 g, 10 mmol) was dissolved in MeCN (40 mL) and cooled to 0 °C. TEA (2.02 g, 20 mmol) was added and the solution was stirred for 5 min. Di-*tert*-butyl dicarbonate (Boc_2O ; 2.18 g, 10 mmol) was dissolved in MeCN (10 mL) and added dropwise to the reaction mixture which was allowed to stir overnight at room temperature. The

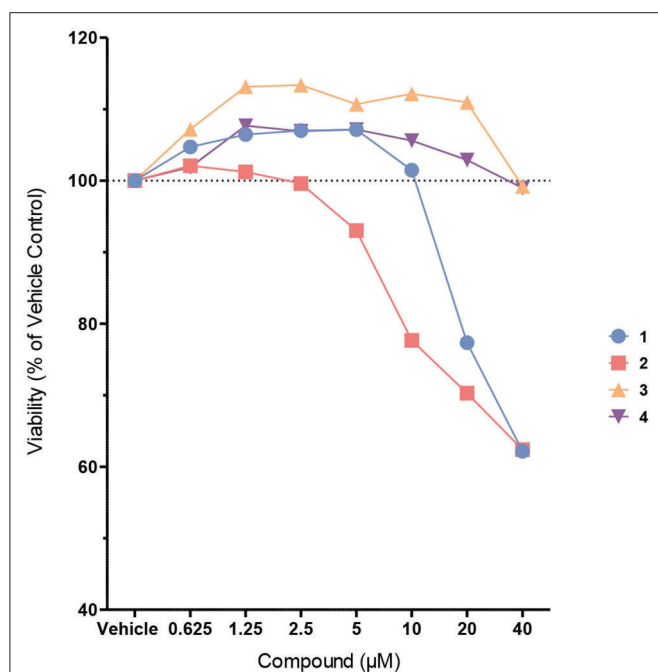


FIGURE 4 | Cell viability of immortalized murine bone marrow-derived macrophages after treatment with probes 1–4. Cells were treated with the probes at different concentrations in Opti-MEM for 4 h. Cell viability was assessed using the CellTiter-Blue cell viability assay.

volatiles were evaporated and the residue was taken up in EtOAc (60 mL). The organic layer was washed with H₂O (3 × 60 mL) and brine (60 mL), dried over Na₂SO₄, filtered and concentrated to yield the product as a white solid. Yield 89%; mp: 144–146 °C; *R*_f = 0.42 (petroleum ether/EtOAc 1+1); ¹H NMR (600 MHz, DMSO-*d*₆) δ 1.39 (s, 9H), 4.18 (d, *J* = 6.1 Hz, 2H), 7.28 (s, 2H), 7.40 (d, *J* = 8.0 Hz, 2H), 7.46 (t, *J* = 6.2 Hz, 1H), 7.76 (d, *J* = 8.2 Hz, 2H); ¹³C NMR (151 MHz, DMSO-*d*₆) δ 28.20, 43.05, 77.98, 125.64, 127.17, 142.50, 144.23, 155.77; LC-MS (ESI) (90% H₂O to 100% MeOH in 10 min, then 100% MeOH to 20 min, DAD 220–400 nm), *t*_R = 8.45 min, 100% purity, *m/z* [M+H]⁺ calcd for C₁₂H₁₈N₂O₄S 287.1, found 287.0.

***tert*-Butyl (4-(*N*-([1,2,3,5,6,7-Hexahydro-*s*-indacen-4-yl]carbamoyl)sulfamoyl)benzyl)carbamate (9)**

Compound **8** (0.46 g, 1.6 mmol) was dissolved in dry THF (15 mL). NaH (60% dispersion in mineral oil; 0.12 g, 2.9 mmol) was added in one portion and the reaction mixture was stirred at 0 °C under nitrogen atmosphere for 30 min. A solution of compound **6** (0.32 g, 1.6 mmol) in dry THF (10 mL) was added and the reaction mixture was allowed to stir at room temperature under nitrogen atmosphere for 4 h. All volatiles were evaporated. The solid residue was taken up in water (10 mL). The solution was acidified with 2 N HCl and precipitate formed was filtered off, washed with EtOAc (10 mL) and dried under high vacuum to yield a white solid. Yield 48%; mp: >250 °C (decomposition); *R*_f = 0.55 (petroleum ether/EtOAc 1+1); ¹H NMR (600 MHz, DMSO-*d*₆) δ 0.57 (s, 9H), 1.04–1.11 (m,

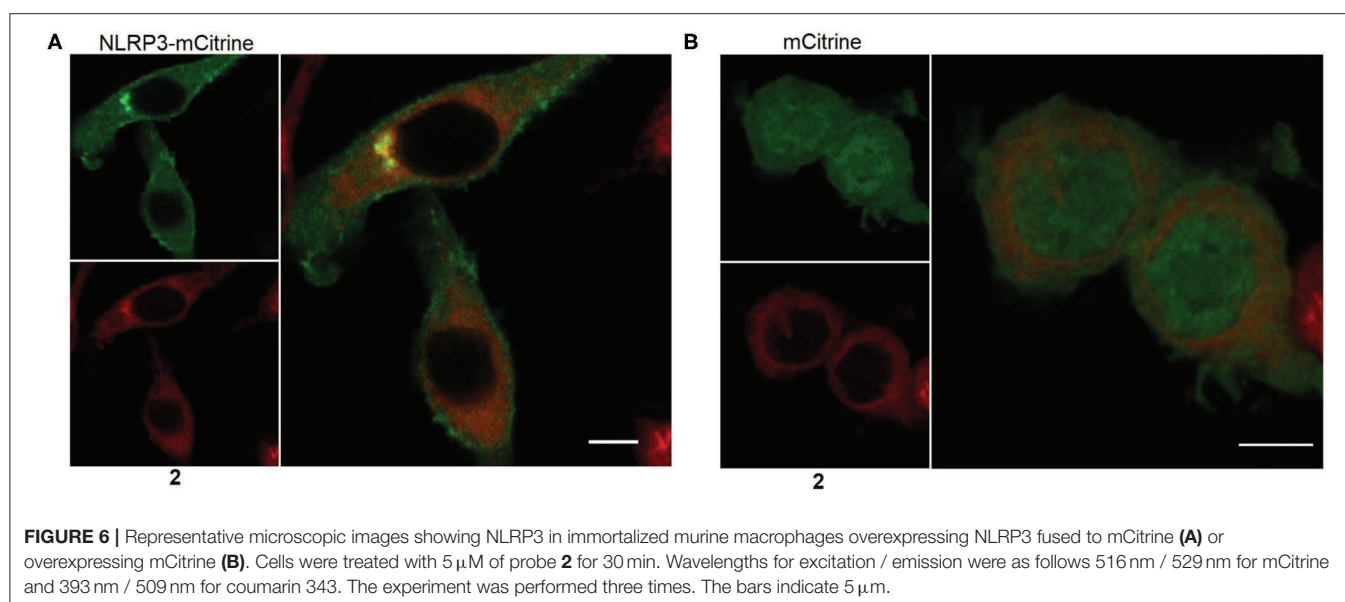
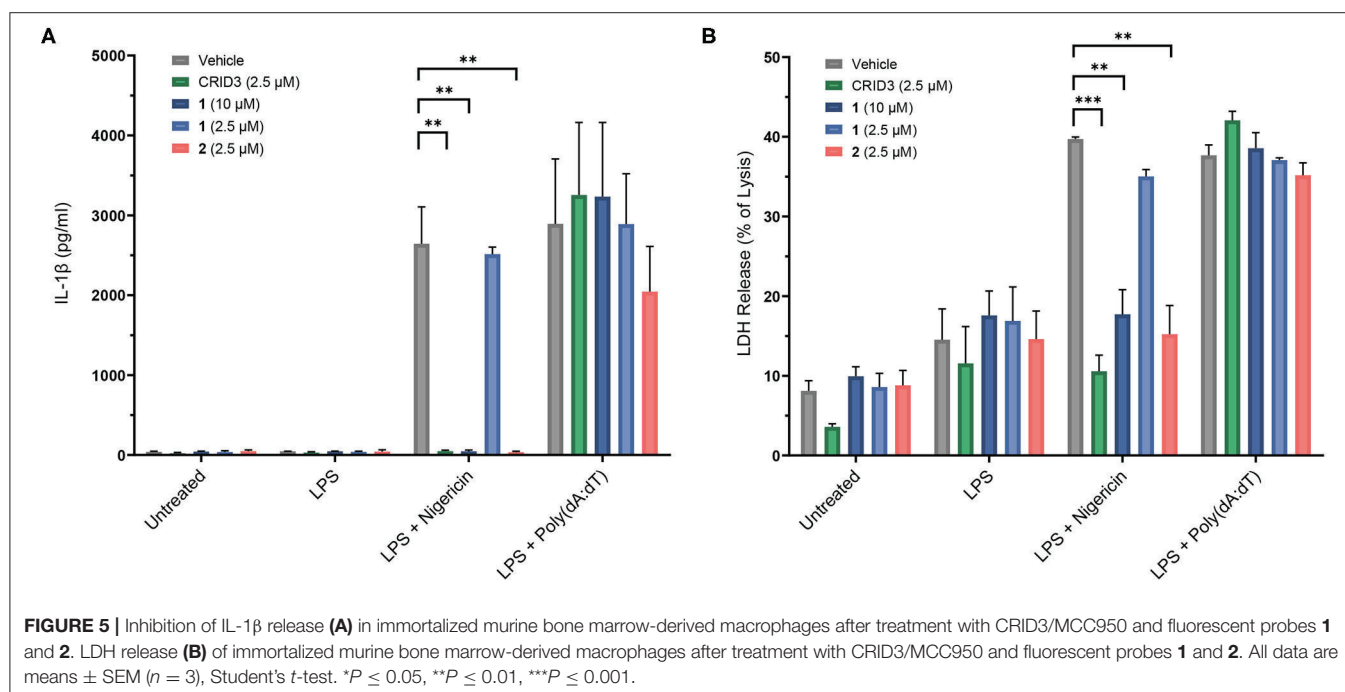
4H), 1.68–1.69 (m, 2H), 1.82 (t, *J* = 7.4 Hz, 4H), 1.92 (t, *J* = 7.4 Hz, 4H), 5.94 (s, 1H), 6.40 (d, *J* = 7.9 Hz, 2H), 6.57 (t, *J* = 6.1 Hz, 1H), 6.63 (s, 1H), 6.88 (d, *J* = 8.0 Hz, 2H), one NH signal is missing due to proton exchange; ¹³C NMR (151 MHz, DMSO-*d*₆) δ 25.04, 28.21, 30.48, 32.56, 43.10, 77.78, 115.49, 126.01, 126.04, 132.66, 136.62, 141.37, 142.06, 145.95, 155.74, 158.43; LC-MS (ESI) (90% H₂O to 100% MeOH in 10 min, then 100% MeOH to 20 min, DAD 220–400 nm), *t*_R = 11.20 min, 100% purity, *m/z* [M+H]⁺ calcd for C₁₅H₃₁N₃O₅S 486.2, found 486.4.

***tert*-Butyl 2-(2-(2-(11-Oxo-2,3,6,7-tetrahydro-1*H*,5*H*,11*H*-pyrano[2,3-*f*]pyrido[3,2,1-*ij*]quinoline-10-carboxamido)ethoxy)ethoxy)acetate (18) (Kohl et al., 2015)**

The orthogonal protected linker *tert*-butyl 3-oxo-1-phenyl-2,7,10-trioxa-4-azadodecan-12-oate (**13**, 0.353 g, 1 mmol; see **Supplementary Material**) was dissolved in dry EtOAc (10 mL) and treated with 10% Pd/C. The suspension was stirred under H₂ (1 atm, balloon) overnight. The mixture was filtered through celite and the filtrate was concentrated to yield the deprotected linker **14**. 11-Oxo-2,3,6,7-tetrahydro-1*H*,5*H*,11*H*-pyrano[2,3-*f*]pyrido[3,2,1-*ij*]quinoline-10-carboxylic acid (**17**, 0.285 g, 1 mmol; see **Supplementary Material**) was dissolved in dry DMF (5 mL). HATU (0.418 g, 1.1 mmol) and DIPEA (0.388 g, 3 mmol) were added. The mixture was stirred for 30 min under argon atmosphere. Then, the deprotected linker **14**, dissolved in DMF (5 mL) was added. The reaction mixture was stirred for 4 h at room temperature. The solution was concentrated under high vacuum. The crude product was purified via silica gel flash column chromatography using a gradient from petroleum ether (100%) to EtOAc (100%) to yield an orange oil. Yield 71%; *R*_f = 0.43 (EtOAc); ¹H NMR (500 MHz, DMSO-*d*₆) δ 1.41 (s, 9H), 1.84–1.92 (m, 4H), 2.69–2.76 (m, 4H), 3.31–3.35 (m, 4H), 3.44–3.48 (m, 2H), 3.52–3.55 (m, 2H), 3.55–3.61 (m, 4H), 3.99 (s, 2H), 7.25 (s, 1H), 8.52 (s, 1H), 8.78 (t, *J* = 5.5 Hz, 1H); ¹³C NMR (126 MHz, DMSO-*d*₆) δ 19.55 (two carbons), 20.50, 26.76, 27.69, 38.74, 48.96, 49.49, 68.16, 69.01, 69.53, 69.83, 80.54, 104.59, 107.32, 107.81, 119.39, 127.10, 147.48, 147.97, 152.05, 161.79, 162.41, 169.28; LC-MS (ESI) (90% H₂O to 100% MeOH in 10 min, then 100% MeOH to 20 min, DAD 220–500 nm), *t*_R = 11.70 min, 94% purity, *m/z* [M+H]⁺ calcd for C₂₆H₃₄N₂O₇ 487.2, found 487.3.

***N*-(2-(2-(2-(4-(*N*-([1,2,3,5,6,7-Hexahydro-*s*-indacen-4-yl]carbamoyl)sulfamoyl)benzyl)amino)-2-oxoethoxy)ethoxy)ethyl)-11-oxo-2,3,6,7-tetrahydro-1*H*,5*H*,11*H*-pyrano[2,3-*f*]pyrido[3,2,1-*ij*]quinoline-10-carboxamide (1)**

Compound **18** (0.15 g, 0.3 mmol) was dissolved in dry CH₂Cl₂ (3 mL) and trifluoroacetic acid (TFA; 3 mL) was added. The solution was stirred for 2 h. After evaporation of all volatiles, the deprotected acid compound was dried under high vacuum. Compound **9** (0.15 g, 0.3 mmol) was dissolved in a mixture of dry CH₂Cl₂ (3 mL) and TFA (3 mL) and allowed to stir 2 h. After evaporation of all volatiles, the resulting trifluoroacetate salt **10**



was dried under high vacuum. The deprotected acid compound was dissolved in dry DMF (5 mL). Under argon atmosphere, HATU (0.13 g, 0.33 mmol), DIPEA (0.12 g, 0.9 mmol) and a solution of the trifluoroacetate salt **10** in dry DMF (5 mL) were added. It was allowed to stir for 4 h, before it was evaporated under high vacuum. The crude product was purified via silica gel flash column chromatography with a gradient from CH₂Cl₂ to CH₂Cl₂/MeOH (8+2) to yield a yellow solid. Yield 51%; mp: 150–152°C; $R_f = 0.45$ (CH₂Cl₂/MeOH 9+1); ¹H NMR (600 MHz, DMSO-*d*₆) δ 1. –1.92 (m, 8H), 2.51–2.54 (m, 4H, signal is mainly obscured by DMSO signal), 2.65 (t, $J = 6.4$ Hz, 2H), 2.69 (t, $J =$

6.2 Hz, 2H), 2.74 (t, $J = 7.4$ Hz, 4H), 3.27–3.29 (m, 4H, signal is mainly obscured by H₂O signal), 3.44–3.47 (m, 2H), 3.52–3.56 (m, 2H), 3.60–3.65 (m, 4H), 3.97 (s, 2H), 4.42 (d, $J = 6.2$ Hz, 2H), 6.90 (s, 1H), 7.22 (s, 1H), 7.48 (d, 2H), 7.87 (d, 2H), 8.05 (s, 1H), 8.31 (t, $J = 6.3$ Hz, 1H), 8.50 (s, 1H), 8.83 (t, $J = 5.5$ Hz, 1H), 10.70 (s, 1H); ¹³C NMR (151 MHz, DMSO-*d*₆) δ 19.48, 19.51, 20.49, 24.94, 26.77, 30.04, 32.38, 38.69, 41.37, 48.93, 49.50, 69.00, 69.33, 70.03, 70.26, 104.58, 107.36, 107.71, 117.90, 119.43, 127.10, 127.23, 127.43, 128.58, 137.10, 138.44, 143.01, 145.17, 147.52, 147.99, 149.02, 152.04, 161.97, 162.41, 169.45; LC-MS (ESI) (90% H₂O to 100% MeOH in 10 min, then 100% MeOH to 20 min,

DAD 220–500 nm), t_R = 11.68 min, 97% purity, m/z calcd for $C_{42}H_{47}N_5O_9S$ 798.3, found 798.7; HRMS(ESI) m/z $[M+Na]^+$ calcd 820.2987, found 820.2990.

***tert*-Butyl 2-(2-(2-(5-([3aR,4R,6aS]-2-Oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamido)ethoxy)ethoxy)acetate (21)**

The orthogonal protected linker *tert*-butyl 3-oxo-1-phenyl-2,7,10-trioxa-4-azadodecan-12-oate (**13**, 0.53 g, 1.5 mmol; see **Supplementary Material**) was dissolved in dry EtOH (10 mL) and treated with 10% Pd/C. The reaction mixture was stirred under H_2 (1 atm, balloon) overnight. The mixture was filtered through celite and the filtrate was concentrated to yield the deprotected linker **14**. 2,5-Dioxopyrrolidin-1-yl 5-([3aR,4R,6aS]-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanoate (**20**, 0.51 g, 1.5 mmol; see **Supplementary Material**) was dissolved in dry DMF (5 mL) and dry CH_2Cl_2 (5 mL) under argon atmosphere. TEA (0.46 g, 4.5 mmol) and the deprotected linker **14**, dissolved in DMF (5 mL) were added. The combined mixture was stirred overnight at room temperature. The solution was evaporated under high vacuum. The crude product was purified via silica gel column chromatography using CH_2Cl_2 /MeOH (9+1) as eluent to yield a colorless resin. Residues of *N*-hydroxysuccinimide (NHS) were removed in the next step. Yield 37%; R_f = 0.23 (CH_2Cl_2 /MeOH 9+1); ^1H NMR (500 MHz, DMSO- d_6) δ 1.24–1.35 (m, 2H), 1.42 (s, 9H), 1.45–1.56 (m, 3H), 1.56–1.67 (m, 1H), 2.06 (t, J = 7.3 Hz, 2H), 2.60–2.62 (m, 1H, signal is mainly obscured by NHS signal), 2.82 (dd, J = 12.4, 5.2 Hz, 1H), 3.07–3.12 (m, 1H), 3.17–3.20 (m, 2H), 3.39–3.41 (m, 2H), 3.51–3.58 (m, 4H), 3.97–3.98 (m, 2H), 4.10–4.16 (m, 1H), 4.29–4.32 (m, 1H), 6.32 (s, 1H), 6.38 (s, 1H), 7.78 (t, J = 5.5 Hz, 1H); ^{13}C NMR (126 MHz, DMSO- d_6) δ 25.18, 27.74, 27.99, 28.14, 35.06, 38.41, 55.36, 59.17, 61.00, 68.11, 69.10, 69.42, 69.82, 80.62, 162.64, 172.07, 172.67. One signal is missing solvent peak; LC-MS (ESI) (90% H_2O to 100% MeOH in 10 min, then 100% MeOH to 20 min, DAD 200–400 nm), t_R = 9.19 min, m/z $[M+H]^+$ calcd for $C_{20}H_{35}N_3O_6S$ 446.2, found 446.2.

***N*-(2-(2-(2-(4-(*N*-([1,2,3,5,6,7-Hexahydro-*s*-indacen-4-yl]carbamoyl)sulfamoyl)benzyl]amino)-2-oxoethoxy)ethoxy)ethyl)-5-([3aR,4R,6aS]-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamide (3)**

Compound **21** (0.27 g, 0.6 mmol) was dissolved in dry CH_2Cl_2 (5 mL) and TFA (5 mL) was added. The solution was stirred for 2 h. After evaporation of all volatiles, the deprotected acid compound was dried under high vacuum. Compound **9** (0.29 g, 0.6 mmol) was dissolved in a mixture of dry CH_2Cl_2 (5 mL) and TFA (5 mL) and allowed to stir 2 h. After evaporation of all volatiles, the resulting trifluoroacetate salt **10** was dried under high vacuum. The deprotected acid compound was dissolved in dry DMF (5 mL). Under argon atmosphere, HATU (0.25 g, 0.66 mmol), DIPEA (0.23 g, 1.8 mmol) and a solution of the trifluoroacetate salt **10** in dry DMF (5 mL) were added. It was allowed to stir for 4 h, before it was evaporated under high vacuum. The crude product was purified via silica gel flash column chromatography with a gradient from CH_2Cl_2 to

CH_2Cl_2 /MeOH (8+2) to yield a white solid. Yield 19%; mp: 140–142°C; R_f = 0.20 (CH_2Cl_2 /MeOH 9+1); ^1H NMR (600 MHz, DMSO- d_6) δ 1.24–1.32 (m, 2H), 1.42–1.53 (m, 3H), 1.57–1.63 (m, 1H), 1.92 (p, J = 7.4 Hz, 4H), 2.05 (t, J = 7.4 Hz, 2H), 2.54 (t, J = 7.4 Hz, 4H), 2.57 (d, J = 12.3 Hz, 1H), 2.77 (t, J = 7.4 Hz, 4H), 2.81 (dd, J = 12.4, 5.1 Hz, 1H), 3.05–3.10 (m, 1H), 3.18 (q, J = 5.8 Hz, 2H), 3.40 (t, J = 5.9 Hz, 2H), 3.54–3.63 (m, 4H), 3.96 (s, 2H), 4.10–4.13 (m, 1H), 4.28–4.31 (m, 1H), 4.39 (d, J = 6.2 Hz, 2H), 6.33 (s, 1H), 6.38 (s, 1H), 6.92 (s, 1H), 7.47 (d, J = 8.2 Hz, 2H), 7.80 (t, J = 5.7 Hz, 1H), 7.87 (d, 2H), 8.06 (s, 1H), 8.36 (t, J = 6.3 Hz, 1H), 10.71 (s, 1H); ^{13}C NMR (151 MHz, DMSO- d_6) δ 24.98, 25.21, 28.00, 28.15, 30.07, 32.41, 35.06, 38.38, 41.36, 55.37, 59.17, 61.00, 69.14, 69.33, 70.00, 70.26, 117.86, 127.23, 127.49, 128.74, 137.11, 138.75, 143.01, 145.00, 149.35, 162.66, 169.53, 172.13. One signal is missing (overlapping solvent peak); LC-MS (ESI) (90% H_2O to 100% MeOH in 10 min, then 100% MeOH to 20 min, DAD 220–400 nm), t_R = 9.86 min, 96% purity, m/z $[M+H]^+$ calcd for $C_{36}H_{48}N_6O_8S_2$ 757.3, found 757.9; HRMS (ESI) m/z $[M+H]^+$ calcd 757.3048, found 757.3049.

Biochemical and Cell Biological Procedures

Expression of Recombinant NLRP3 Protein

HEK293T cells were transiently co-transfected with human avi-FLAG-His10-NLRP3-PYD-NACHT domain (pIRES puro 3 vector) and BirA (pcDNA3.1 vector) for in-cell biotinylation at the lysine amino group of the Avi-tag. After 24 h expression, cells were chemically disrupted and avi-FLAG-His10-NLRP3-PYD-NACHT domain was isolated using anti-FLAG M2 affinity beads (Sigma Aldrich). Eluted protein was stored snap frozen in liquid nitrogen, aliquoted, and stored at $-80^\circ C$.

Surface Plasmon Resonance Spectroscopy

SPR experiments were performed using a Biacore 8K instrument (GE Healthcare). The flow system was cleaned using the maintenance “Desorb” function (Desorb Kit, GE Healthcare). The system was flushed with running buffer (10 mM HEPES pH 7.4, 200 mM NaCl, 0.5 mM ADP, 0.5 mM tris(2-carboxyethyl)phosphine (TCEP), 2 mM $MgCl_2$, 1 g/L carboxymethyl dextran hydrogel (CMD), 0.05% Tween20, 2% DMSO) and all steps were performed at $25^\circ C$ chip temperature. Before immobilization, a streptavidin functionalized sensor chip (Series S Sensor Chip SA, GE Healthcare) was conditioned with three consecutive 1-min injections of 1 M NaCl in 50 mM NaOH (10 μL /min). Biotinylated NLRP3-PYD-NACHT protein (2 μL /min, 900 s) was immobilized on the sensor chip flow cell 2. The flow system was washed using 50% isopropanol in 1 M NaCl and 50 mM NaOH. Free streptavidin binding sites were blocked by four consecutive 2-min injections (1,000 nM, 10 μL /min) of Biotin-PEG (Mn = 2,300 Da) (Sigma Aldrich). For kinetic binding measurements, different concentrations of CRID3/MCC950 (31.25 nM, 62.5 nM, 125 nM, 150 nM, 300 nM, 600 nM) or test compounds **1–4** (312.5 nM, 625 nM, 1,250 nM, 1,500 nM, 3,000 nM, 6,000 nM) were injected (30 μL /min, association: 120 s, dissociation: 60/360 s) over both flow

cells. For competition measurements, the first compound was injected (30 μ l/min, monitored over 120 s) at a concentration 10-times the kinetic K_D of test compound **2** or **4**, respectively. In a second injection step, equimolar concentration of the second compound was injected (30 μ l/min, 120 s) in presence of the first compound. Data were collected at a rate of 10 Hz. The binding data were double referenced by blank cycle and reference flow cell subtraction. Data were corrected by a 4-point solvent correction. For kinetic binding experiments, processed data were fitted to a 1:1 interaction model using the Biacore Insight Evaluation Software (version 3.0.12.15655).

Cell Culture

Immortalized murine bone marrow-derived macrophages were used (De Nardo et al., 2018). Cells were cultured in T-75 flasks with complete Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin in a humidified incubator at 37°C with 5% CO₂.

Cell Viability Assay

NLRP3 KO immortalized murine bone marrow-derived macrophages overexpressing mouse NLRP3-FLAG and human ASC-mCerulean were seeded in 96 well plates and incubated overnight. Cells were treated with the fluorescent probes (**1** and **2**) and the biotin-tagged probes (**3** and **4**) at different concentrations in Opti-MEM for 4 h. Subsequently, cell viability was assessed using CellTiter-Blue[®] cell viability assay according to the manufacturer's instructions.

Inflammasome and LDH Release Assays

NLRP3 KO Immortalized murine bone marrow-derived macrophages overexpressing mouse NLRP3-Flag and human ASC-mCerulean were seeded in 96-well plates and incubated for 16 h. Cells were treated with 400 ng/mL ultrapure LPS for 3 h. After priming, the probes or CRID3/MCC950 at various concentrations or DMSO were added to the cells for 30 min. Subsequently, to induce NLRP3 inflammasome activation, cells were treated with 10 μ M nigericin for 1.5 h. As controls, to activate the AIM2 inflammasome, cells were transfected with 200 ng of poly(dA:dT) using 0.5 μ L lipofectamine 2,000 per well for 4 h. Supernatants were collected to measure cytosolic LDH release by an LDH assay and IL-1 β release by Homogeneous

Time Resolved Fluorescence (HTRF). All assays were performed according to the manufacturer's instructions. A two-tailed unpaired *t*-test was performed using GraphPad Prism 9.

Confocal Microscopy

Immortalized murine bone marrow-derived macrophages overexpressing NLRP3-mCitrine or mCitrine were seeded in Ibidi μ -slides. After 16 h, the fluorescent probe **2** was diluted in complete DMEM and added to the cells at 5 μ M concentration for 30 min. Cells were fixed with 4% methanol-free formaldehyde for 10 min at room temperature. They were then washed three times with PBS and directly imaged with a Leica SP8 lightning confocal microscope.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

TK and MGü designed compounds and wrote the manuscript. TK synthesized compounds. KG and MM performed SPR experiments. AA and ML performed cellular assays and microscopy. All authors analyzed data. MGe, EL, and MGü supervised the study. TK and KG contributed equally. MGü conceived the study.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fchem.2021.642273/full#supplementary-material>

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary Material

SUPPLEMENTARY DATA

Syntheses

N-(4-(*N*-((1,2,3,5,6,7-hexahydro-*s*-indacen-4-yl)carbamoyl)sulfamoyl)benzyl)-11-oxo-2,3,6,7-tetrahydro-1*H*,5*H*,11*H*-pyrano[2,3-*f*]pyrido[3,2,1-*ij*]quinoline-10-carboxamide (**2**). The Boc-protected compound *tert*-butyl (4-(*N*-((1,2,3,5,6,7-hexahydro-*s*-indacen-4-yl)carbamoyl)sulfamoyl)benzyl)carbamate (**9**, 0.24 g, 0.5 mmol) was dissolved in dry CH₂Cl₂ (5 mL) and TFA (5 mL) was added. The mixture was stirred at room temperature for 2 h. After removal of the volatiles, the residue was further dried under high vacuum to yield the trifluoroacetate salt **10**. Compound **17** (0.14 g, 0.5 mmol) was dissolved in dry DMF (5 mL) under argon atmosphere. HATU (0.18 g, 0.55 mmol) and DIPEA (0.19 g, 1.5 mmol) were added and it was stirred for 30 min. Then, the entire material of the trifluoroacetate salt **10** was added. The mixture was stirred for 4 h at room temperature, was quenched with H₂O (20 mL), diluted with saturated NH₄Cl solution (50 mL) and extracted with EtOAc (3 × 30 mL). The combined organic phases were washed with sat. NH₄Cl (60 mL), dried over Na₂SO₄, filtered and concentrated. The crude product was purified via silica gel column chromatography using CH₂Cl₂/MeOH (19+1) as eluent. The product fractions were evaporated. The solid residue was resuspended in EtOAc, filtered, washed with MeOH and dried under high vacuum to yield a fine yellow solid. Yield 46%; mp: >250 °C (decomposition); *R*_f = 0.47 (CH₂Cl₂/MeOH 19+1); ¹H NMR (600 MHz, DMSO-*d*₆) δ 1.85 – 1.92 (m, 8H), 2.51 – 2.54 (m, 4H, signal is mainly obscured by DMSO signal), 2.69 – 2.71 (m, 2H), 2.72 – 2.76 (m, 6H), 3.31 – 3.34 (m, 4H, signal is mainly obscured by H₂O signal), 4.60 (d, *J* = 6.2 Hz, 2H), 6.91 (s, 1H), 7.23 (s, 1H), 7.53 (d, *J* = 8.0 Hz, 2H), 7.89 (d, *J* = 8.0 Hz, 2H), 8.06 (s, 1H), 8.51 (s, 1H), 9.17 (t, *J* = 6.1 Hz, 1H), 10.70 (s, 1H); ¹³C NMR (151 MHz, DMSO-*d*₆) δ 19.55, 20.49 (two carbons), 24.95, 26.76, 30.03, 32.38, 42.15, 48.99, 49.52, 104.60, 107.36, 107.64, 117.94, 119.43, 127.15, 127.34, 127.60, 128.56, 137.14, 138.42, 143.02, 145.33, 147.72, 148.07, 149.00, 152.12, 161.78, 162.80; LC-MS (ESI) (90% H₂O to 100% MeOH in 10 min, then 100% MeOH to 20 min, DAD 220–500 nm), *t*_R = 11.81 min, 96% purity, *m/z* [M+H]⁺ calcd for C₃₆H₃₆N₄O₆S 653.2, found 653.5; HRMS (ESI) *m/z* [M+Na]⁺ calcd 675.2248, found 675.2264.

N-(4-(*N*-((1,2,3,5,6,7-hexahydro-*s*-indacen-4-yl)carbamoyl)sulfamoyl)benzyl)-5-((3*aR*,4*R*,6*aS*)-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)pentanamide (**4**). *tert*-Butyl (4-(*N*-((1,2,3,5,6,7-hexahydro-*s*-indacen-4-yl)carbamoyl)sulfamoyl)benzyl)carbamate (**9**, 0.24 g, 0.5 mmol) was dissolved in dry CH₂Cl₂ (5 mL) and treated with TFA (5 mL). The solution was stirred for 2 h at room temperature. Then it was evaporated and dried under high vacuum to give the trifluoroacetate salt **10**. Compound **20** (0.17 g, 0.5 mmol) was dissolved in dry DMF (5 mL). TEA (0.15 g, 1.5 mmol) and a solution of the trifluoroacetate salt **10** in DMF (5 mL) were added. The reaction mixture was allowed to stir overnight at room temperature. The solution was concentrated under high vacuum. The crude product was purified via silica gel column chromatography using CH₂Cl₂/MeOH (9+1) as eluent to yield a white solid. Yield 43%; mp: >230 °C (decomposition); *R*_f = 0.46 (CH₂Cl₂/MeOH 9+1); LC-MS(ESI) (90% H₂O to 100% MeOH in 10 min, then 100% MeOH to 20 min, DAD 220-400 nm), *t*_R = 10.05 min, 96% purity, *m/z* [M+H]⁺ calcd for C₃₀H₃₇N₅O₅S₂ 612.2, found 612.6; HRMS (ESI) *m/z* [M+H]⁺ calcd 612.2309, found 612.2303.

Benzyl (2-(2-hydroxyethoxy)ethyl)carbamate (**12**) (Adamczyk et al., 2002). 2-(2-Aminoethoxy)ethanol (**11**, 7.89 g, 75 mmol) was dissolved in dry CH₂Cl₂ (75 mL). TEA (7.68 g, 75 mmol) was added and the reaction mixture was cooled to 0 °C. *Benzyl* chloroformate (CAB; 12.18 g, 75 mmol) was dissolved in dry CH₂Cl₂ (75 mL) and was slowly added. It was stirred for 2 h at 0 °C and then for 18 h at room temperature. The reaction was quenched with saturated sodium bicarbonate solution (150 mL) and the aqueous layer was extracted with CH₂Cl₂ (3 × 150 mL). The combined organic layers were washed with brine (50 mL), dried over Na₂SO₄, filtered and concentrated. The crude product was purified via silica gel column chromatography using EtOAc as eluent to yield a colorless oil. Yield 88%; *R*_f = 0.48 (EtOAc); ¹H NMR (600 MHz, DMSO-*d*₆) δ 3.16 (q, *J* = 5.9 Hz, 2H), 3.39 – 3.42 (m, 4H), 3.48 (q, *J* = 5.3 Hz, 2H), 4.58 (t, *J* = 5.5 Hz, 1H), 5.01 (s, 2H), 7.27 (t, *J* = 5.8 Hz, 1H), 7.30 – 7.38 (m, 5H); ¹³C NMR (151 MHz, DMSO-*d*₆) δ 40.27, 60.20, 65.24, 69.10, 72.11, 127.77, 127.78, 128.37, 137.21, 156.19; LC-MS (ESI) (90% H₂O to 100% MeOH in 10 min, then 100% MeOH to 20 min, DAD 200-400 nm), *t*_R = 8.37 min, 100% purity, *m/z* [M+H]⁺ calcd for C₁₂H₁₇NO₄ 240.1, found 239.9.

tert-Butyl 3-oxo-1-phenyl-2,7,10-trioxa-4-azadodecan-12-oate (**13**) (Adamczyk et al., 2002). Compound **12** (1.20 g, 5 mmol) was dissolved in dry THF (10 mL) and cooled to 0 °C. A suspension of potassium *tert*-butoxide (0.56 g, 5 mmol) in dry THF (10 mL) was added. The reaction mixture stirred for 30 min at 0 °C. Then *tert*-butyl bromoacetate (1.17 g, 6 mmol) was added in one portion.

The solution was stirred for 3 h at 0 °C and then for 18 h at room temperature. H₂O (5 mL) was added and the mixture was concentrated. The residue was partitioned between EtOAc (50 mL) and H₂O (50 mL) and the aqueous layer was extracted with EtOAc (2×50 mL), dried over Na₂SO₄, filtered and concentrated. The crude product was purified via silica gel column chromatography using petroleum ether/EtOAc (2+1) as eluent to yield a colorless oil. Yield 57%; *R*_f = 0,33 (petroleum ether/EtOAc 2+1); ¹H NMR (600 MHz, DMSO-*d*₆) δ 1.42 (s, 9H), 3.15 (q, *J* = 5.9 Hz, 2H), 3.42 (t, *J* = 6.0 Hz, 2H), 3.50 – 3.57 (m, 4H), 3.97 (s, 2H), 5.01 (s, 2H), 7.24 (t, *J* = 5.7 Hz, 1H), 7.29 – 7.38 (m, 5H); ¹³C NMR (151 MHz, DMSO-*d*₆) δ 27.73, 40.14, 65.18, 68.11, 69.07, 69.42, 69.82, 80.61, 127.68, 127.71, 128.30, 137.17, 156.13, 169.34; LC-MS (ESI) (90% H₂O to 100% MeOH in 10 min, then 100% MeOH to 20 min, DAD 200-400 nm), *t*_R = 10.85 min, 90% purity, *m/z* [M+H]⁺ calcd for C₁₈H₂₇NO₆ 354.2, found 354.1.

*11-Oxo-2,3,6,7-tetrahydro-1H,5H,11H-pyrano[2,3-*f*]pyrido[3,2-*l*-*ij*]quinoline-10-carboxylic acid* (**17**). Isopropylidene malonate (**15**, 3.53 g, 10 mmol) and piperidinium acetate (6.44 g, 0.4 mmol) were added to a solution of 8-hydroxyjulolidine-9-carboxaldehyde (**16**, 4.47 g, 10 mmol) in absolute ethanol (10 mL). The mixture was allowed to stir at room temperature for 20 min and was then heated to reflux for 2 h. The reaction mixture was allowed to cool to room temperature and stored at –18 °C for 2 h. The crystallized solid was filtered off and washed with ice-cooled absolute ethanol (10 mL) to yield a pure orange solid. Yield 41%; mp: 250-252 °C, lit. mp: 253 °C (van Gompel and Schuster, 1987); *R*_f = 0.22 (petroleum ether/EtOAc 1+1 + 1% AcOH); ¹H NMR (600 MHz, DMSO-*d*₆) δ 1.84 – 1.92 (m, 4H), 2.69 – 2.75 (m, 4H), 3.33 – 3.37 (m, 4H), 7.24 (s, 1H), 8.45 (s, 1H), 12.41 (s, 1H); ¹³C NMR (151 MHz, DMSO-*d*₆) δ 19.47 (two carbons), 20.44, 26.73, 49.09, 49.62, 104.71, 105.06, 107.22, 119.51, 127.45, 148.69, 149.15, 152.63, 160.55, 164.59; LC-MS (ESI) (90% H₂O + 0.1% AcOH to 100% MeOH + 0.1% AcOH in 10 min, then 100% MeOH + 0.1% AcOH to 20 min, DAD 220-500 nm), *t*_R = 10.43 min, 99% purity, *m/z* [M+H]⁺ calcd for C₁₆H₁₅NO₄ 286.1, found 285.9 [M+H]⁺.

*2,5-Dioxopyrrolidin-1-yl 5-((3*aR*,4*R*,6*aS*)-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)pentanoate* (**20**). D-Biotin (**19**, 2.44 g, 10 mmol) was dissolved in dry DMF (70 mL) at 70 °C. Then it was allowed to cool to room temperature. DCC (2.06 g, 10 mmol) and pyridine (0.79 g, 10 mmol) were added to the solution and it was stirred for 5 min, before *N*-hydroxysuccinimide (1.50 g, 13 mmol) was added. The reaction mixture was stirred for 18 h at room temperature. The reaction mixture was filtered and concentrated under high vacuum. The residue was dispersed in isopropanol (150 mL) and refluxed for 30 min. The mixture was stored at –18 °C overnight. The precipitate

formed was filtered off and dried under high vacuum to yield a white solid. Yield 85%; mp: 198-200 °C, lit. mp: 199-201 °C (Piggott and Karuso, 2008); $R_f = 0.41$ ($\text{CH}_2\text{Cl}_2/\text{MeOH}$ 9+1); ^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ 1.38 – 1.46 (m, 2H), 1.46 – 1.55 (m, 1H), 1.60 – 1.70 (m, 3H), 2.59 (d, $J = 12.4$ Hz, 1H), 2.67 (t, $J = 7.4$ Hz, 2H), 2.80 – 2.86 (m, 5H), 3.08 – 3.13 (m, 1H), 4.13 – 4.17 (m, 1H), 4.29 – 4.33 (m, 1H), 6.33 (s, 1H), 6.39 (s, 1H); ^{13}C NMR (126 MHz, $\text{DMSO}-d_6$) δ 24.26, 25.40, 27.52, 27.79, 29.97, 55.16, 59.14, 60.96, 162.62, 168.86, 170.18. One signal is missing (overlapping solvent peak); LC-MS (ESI) (90% H_2O to 100% MeCN in 10 min, then 100% MeCN to 20 min, DAD 220-400 nm), $t_R = 5.79$ min, m/z $[\text{M}+\text{H}]^+$ calcd for $\text{C}_{14}\text{H}_{19}\text{N}_3\text{O}_5\text{S}$ 342.1, found 342.2.

SUPPLEMENTARY FIGURES

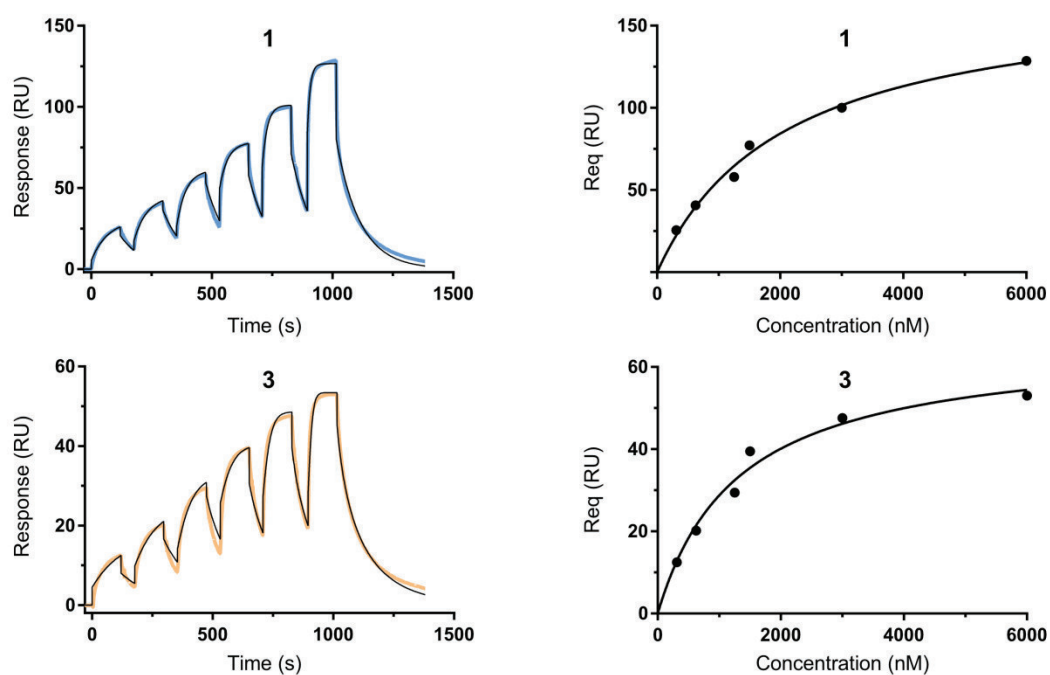


Figure S1. SPR sensorgrams (left) and concentration-binding response unit curves (right) of compound **1** (top) and compound **3** (bottom), injected on the chip surface loaded with human NLRP3-PYD-NACHT. The binding parameters obtained from the sensorgrams are listed in Table 1. Data were fitted to a 1:1 binding model.

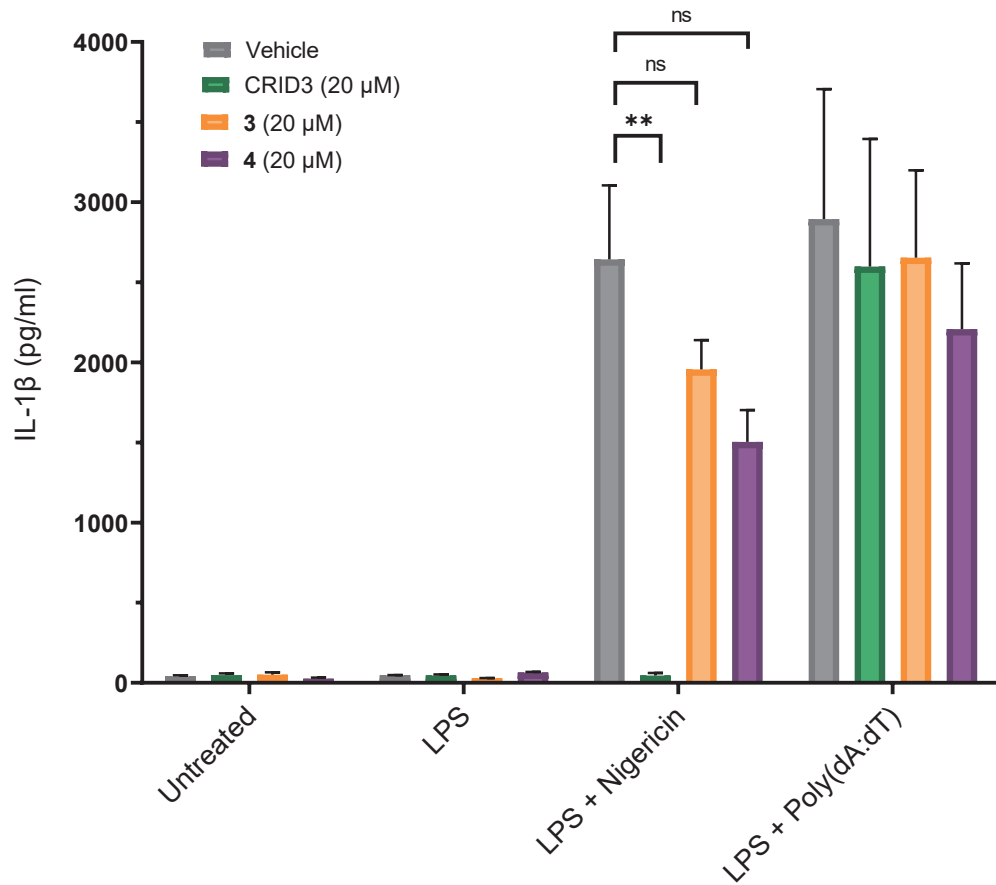


Figure S2. Inhibition of IL-1 β release in bone marrow-derived macrophages after treatment with CRID3/MCC950 and biotin probes **3** and **4**. All data are means $\pm$ SEM (n=3), Student's t-test. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

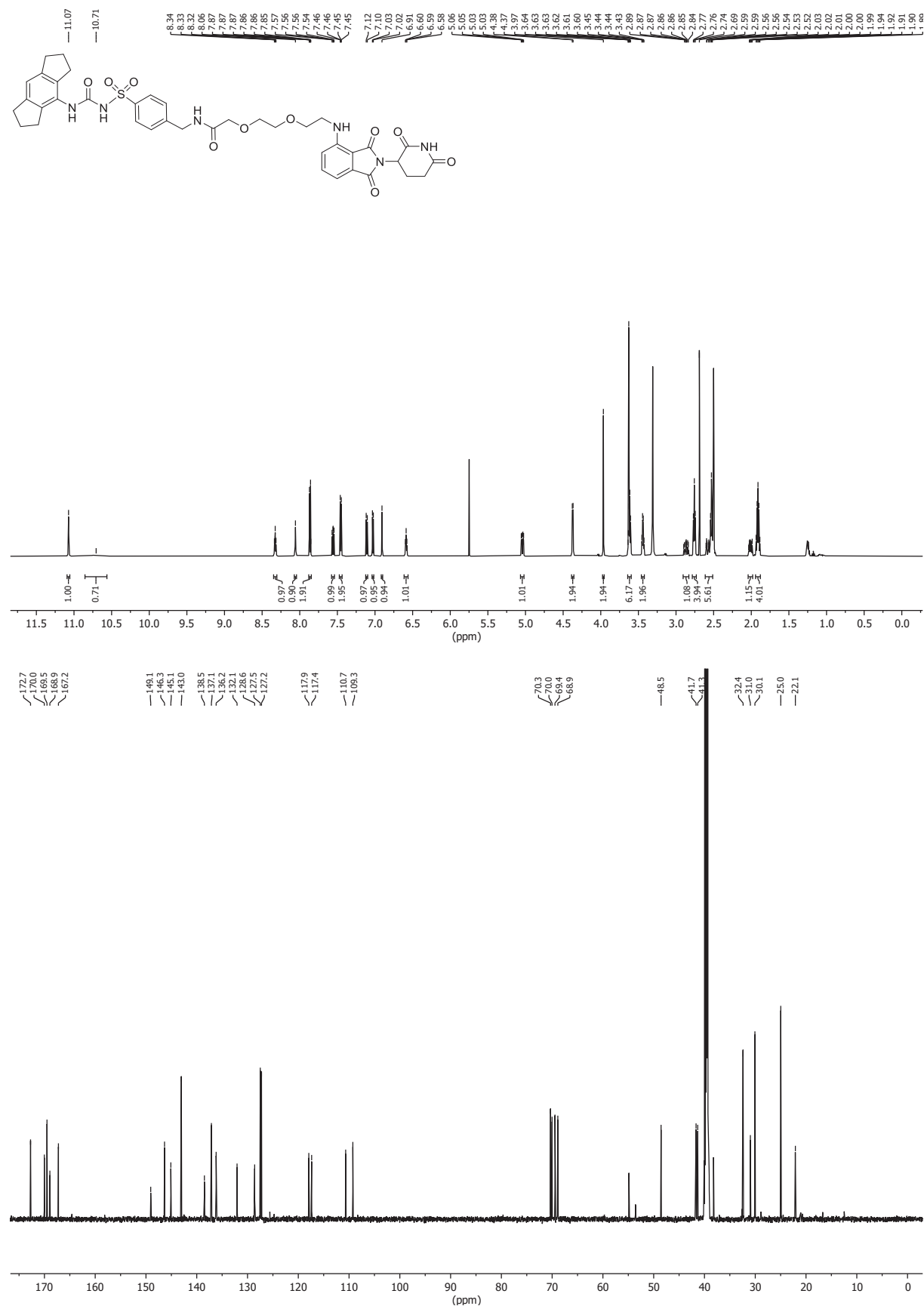
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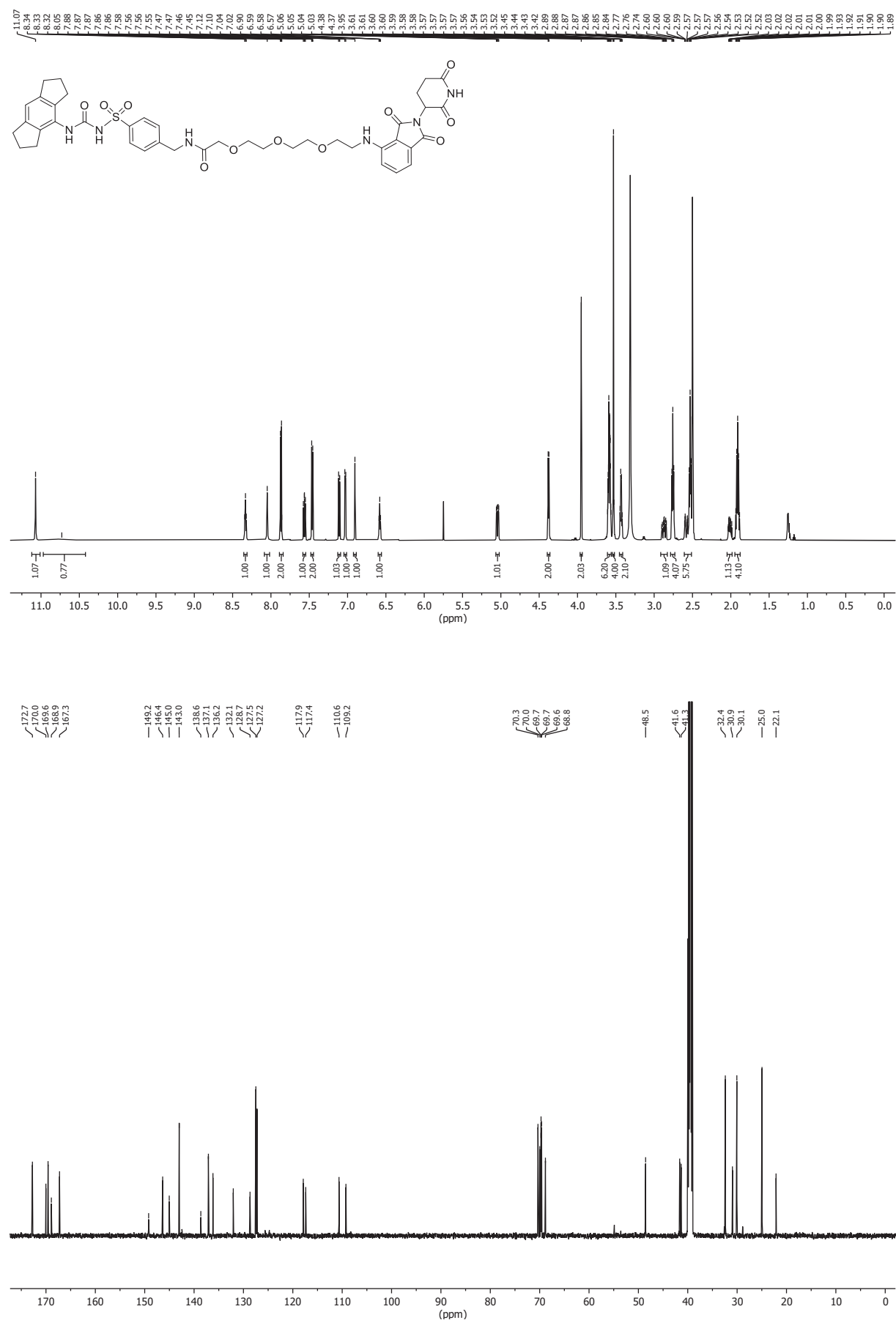
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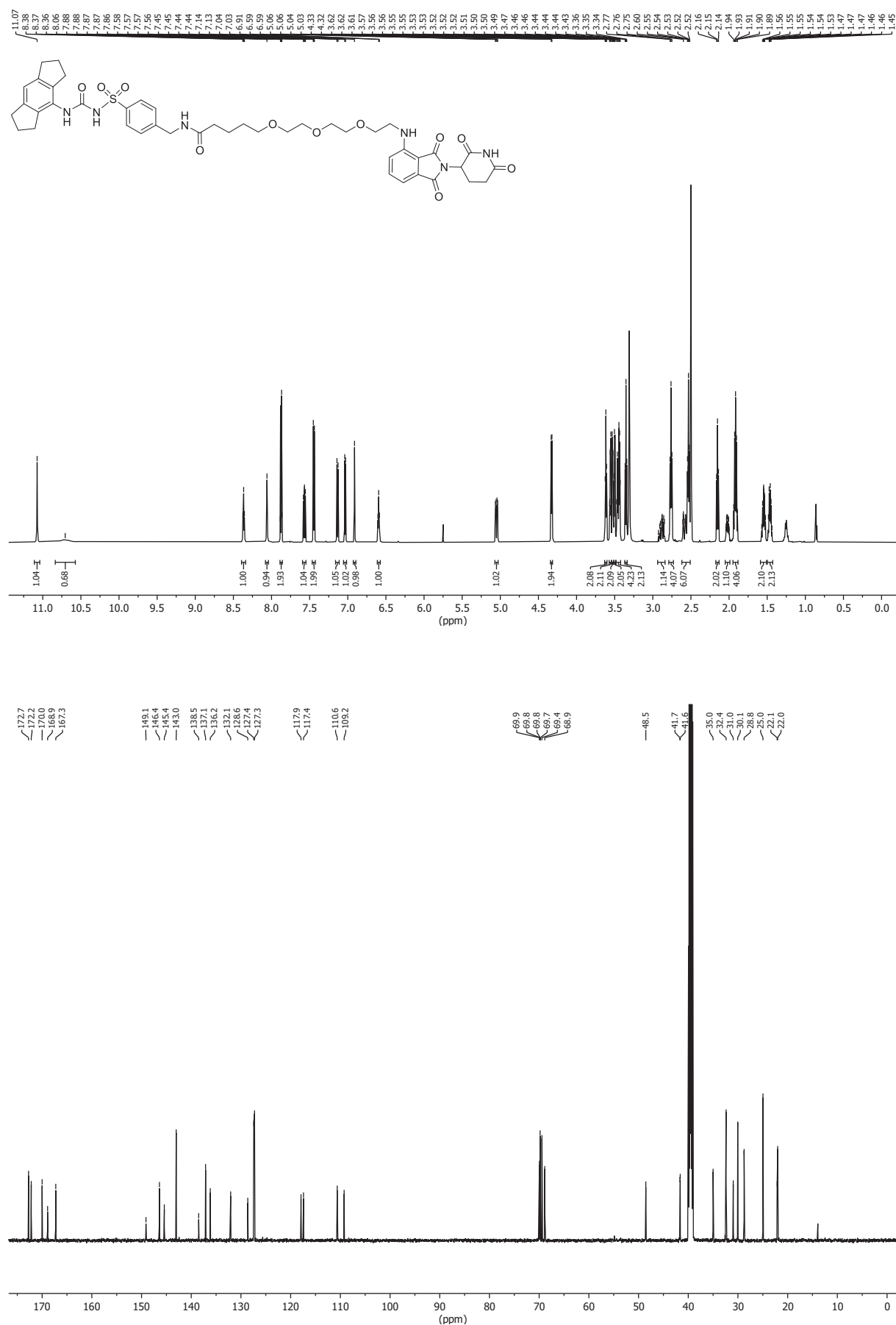


Appendix IV. NMR spectra of Compounds reported in Chapter 5

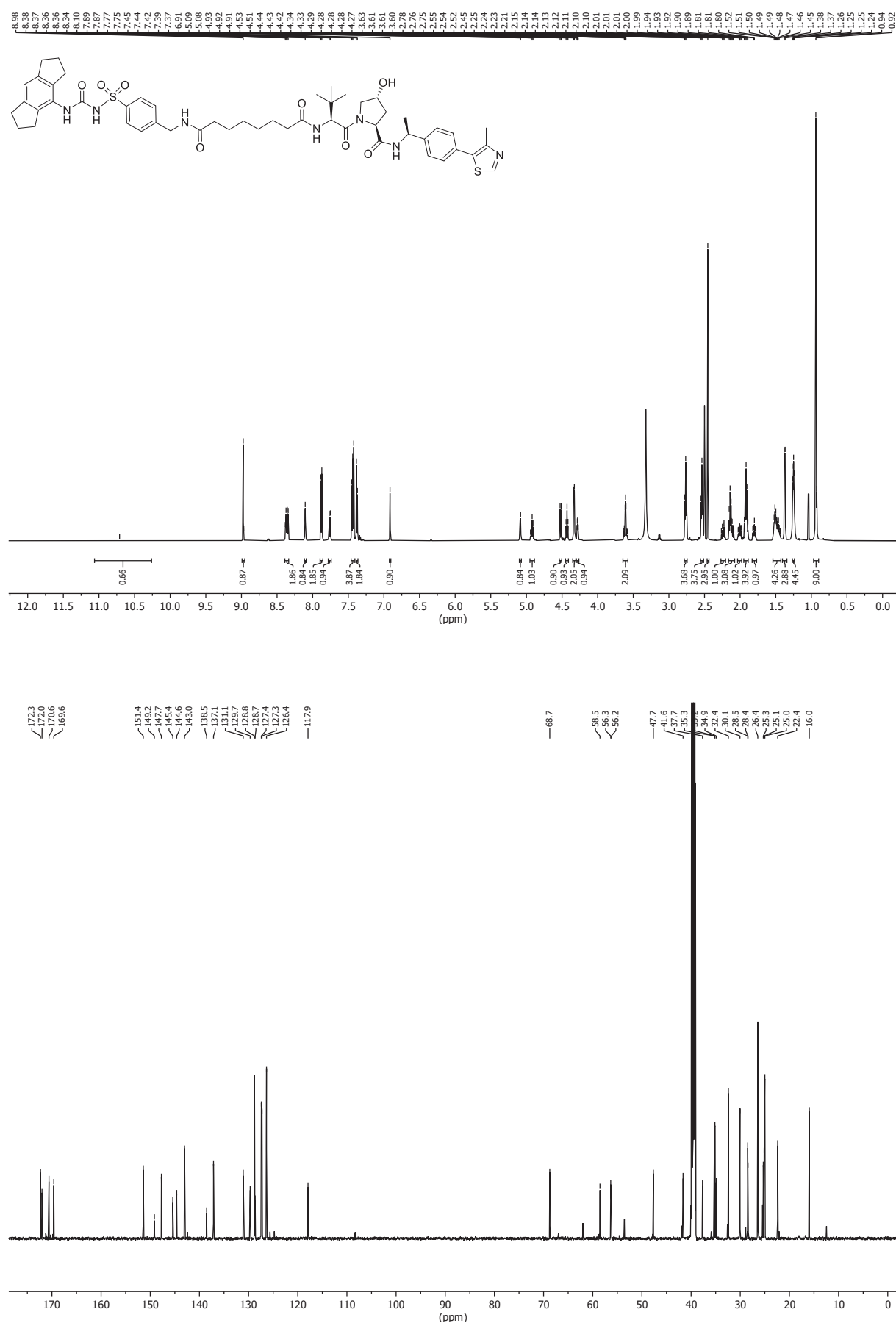
The following pages include ^1H and ^{13}C NMR spectra of final compounds reported in chapter 5.

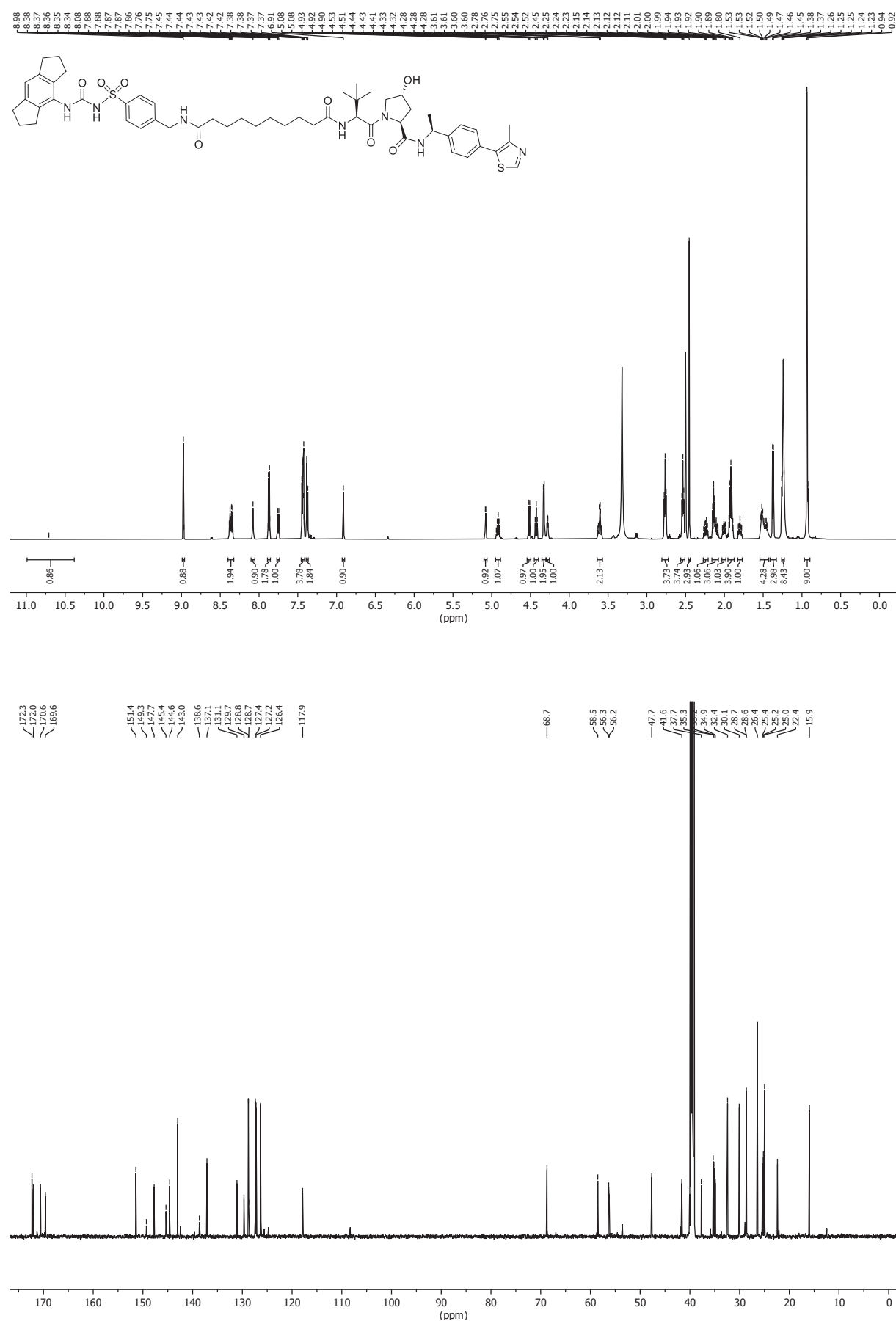
^1H and ^{13}C NMR spectra **^1H and ^{13}C NMR spectra of compound **120****

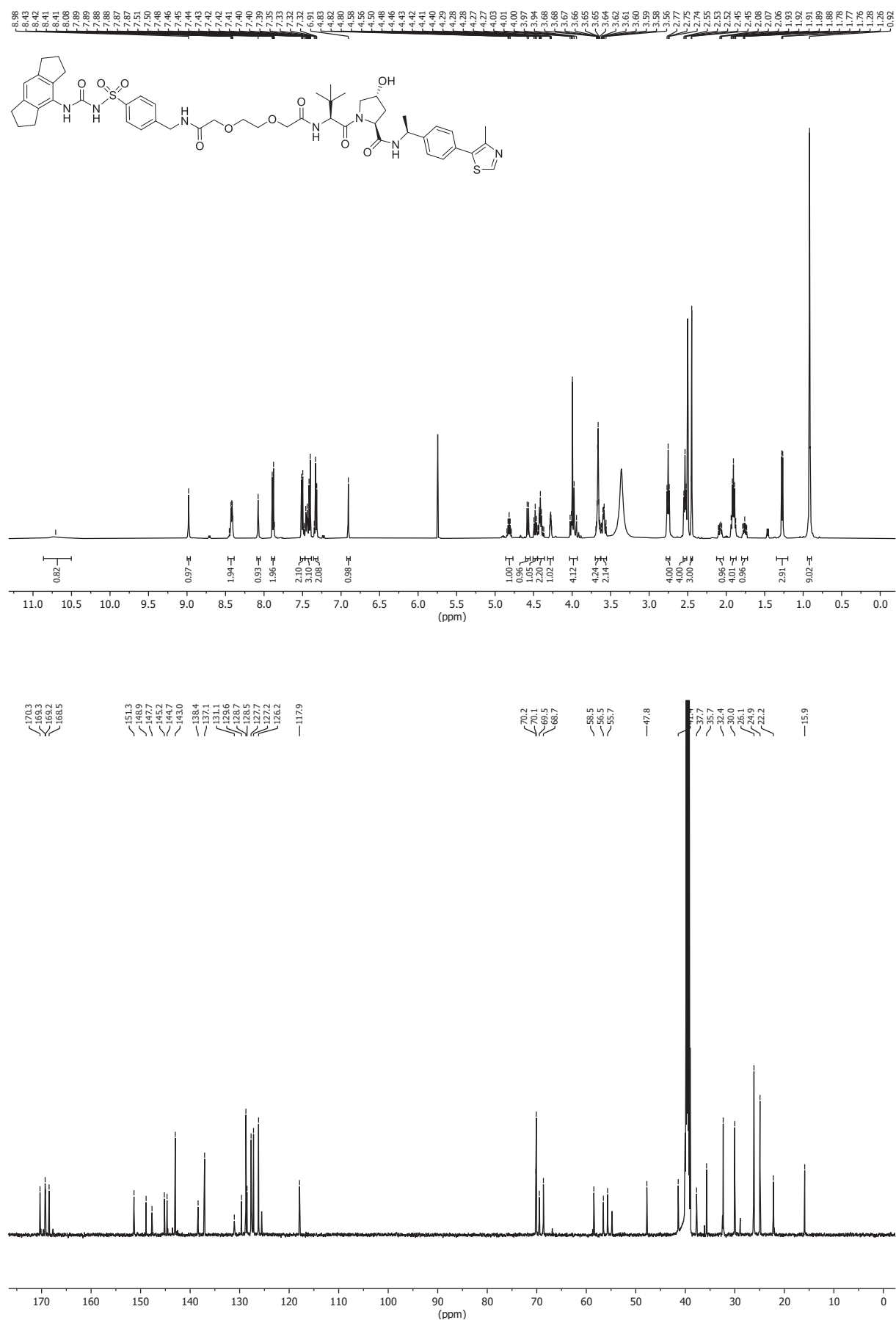
^1H and ^{13}C NMR spectra of compound **121**

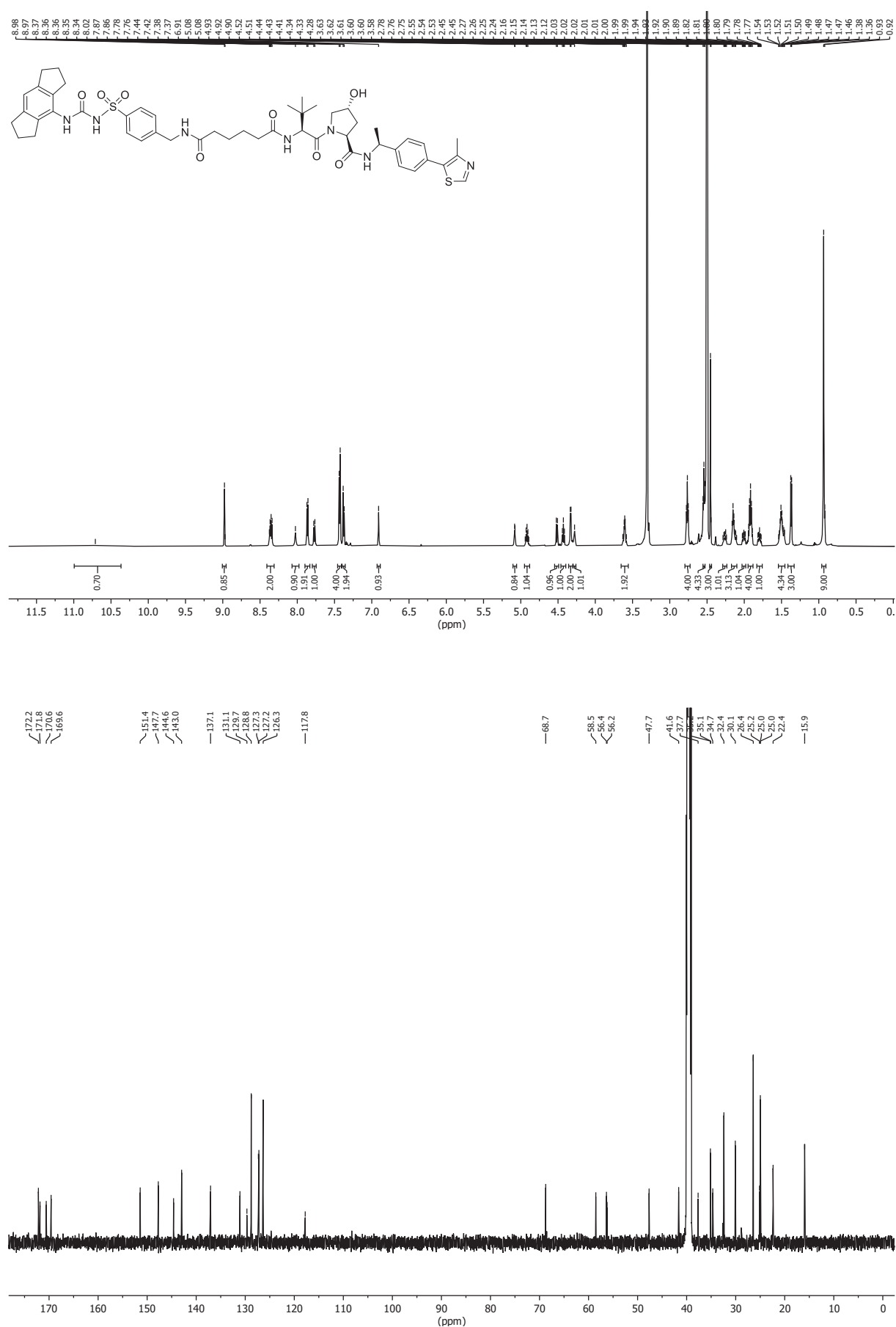
^1H and ^{13}C NMR spectra of compound **122**

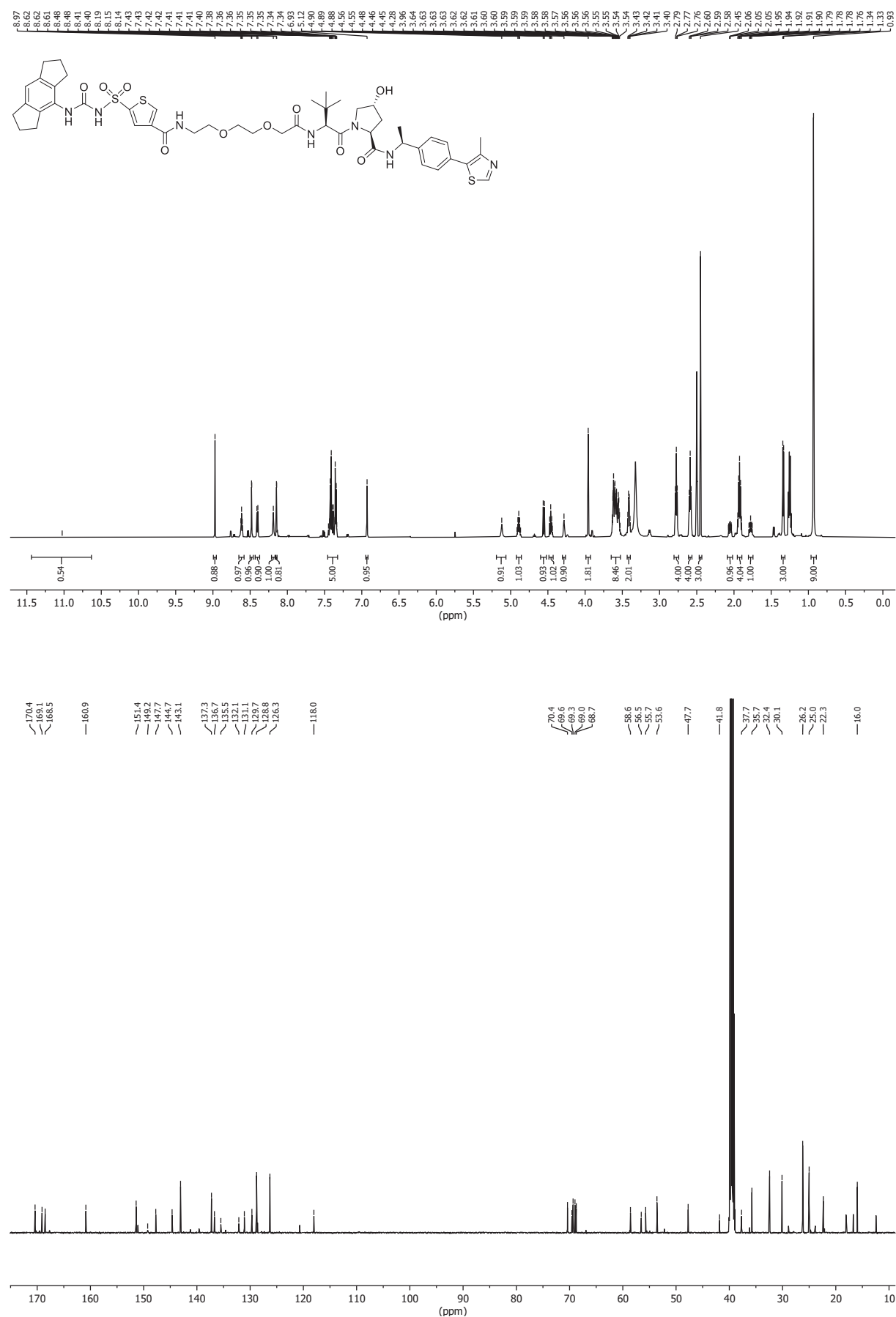


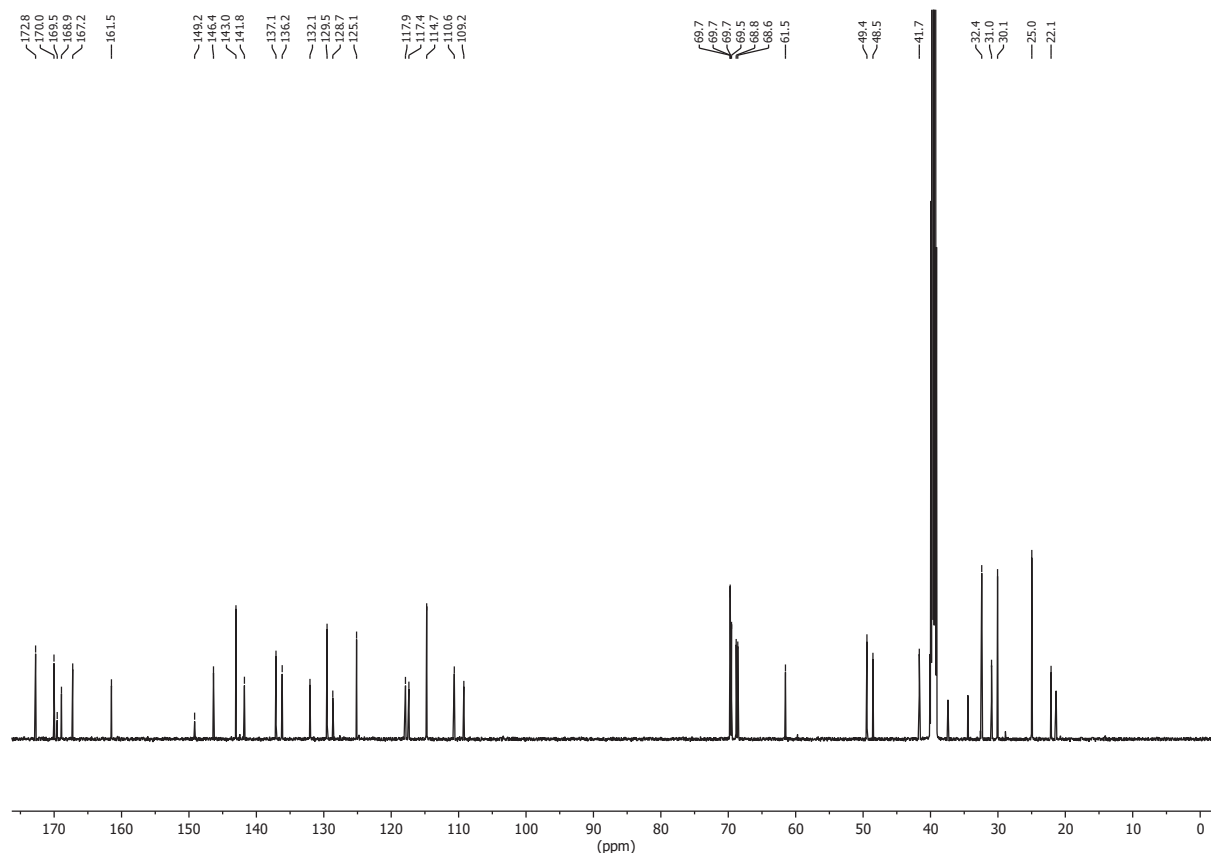
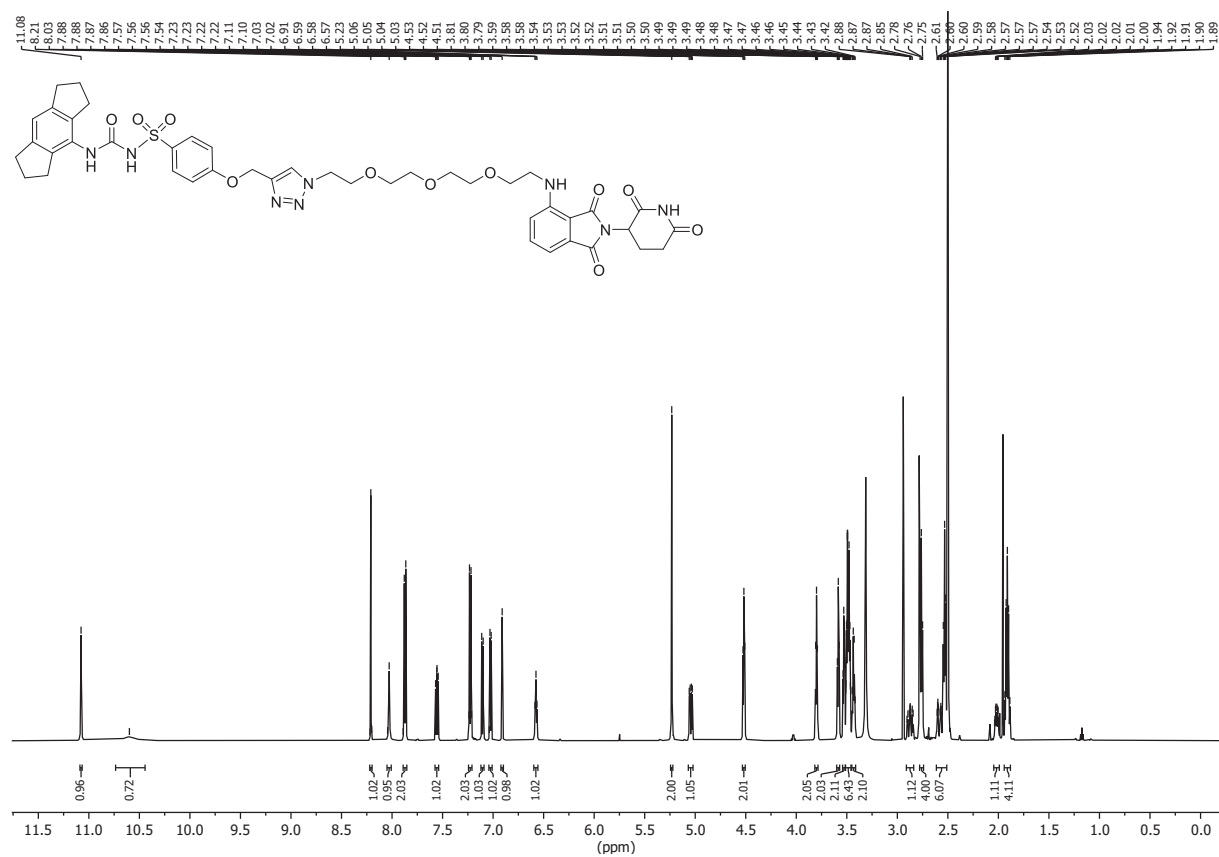
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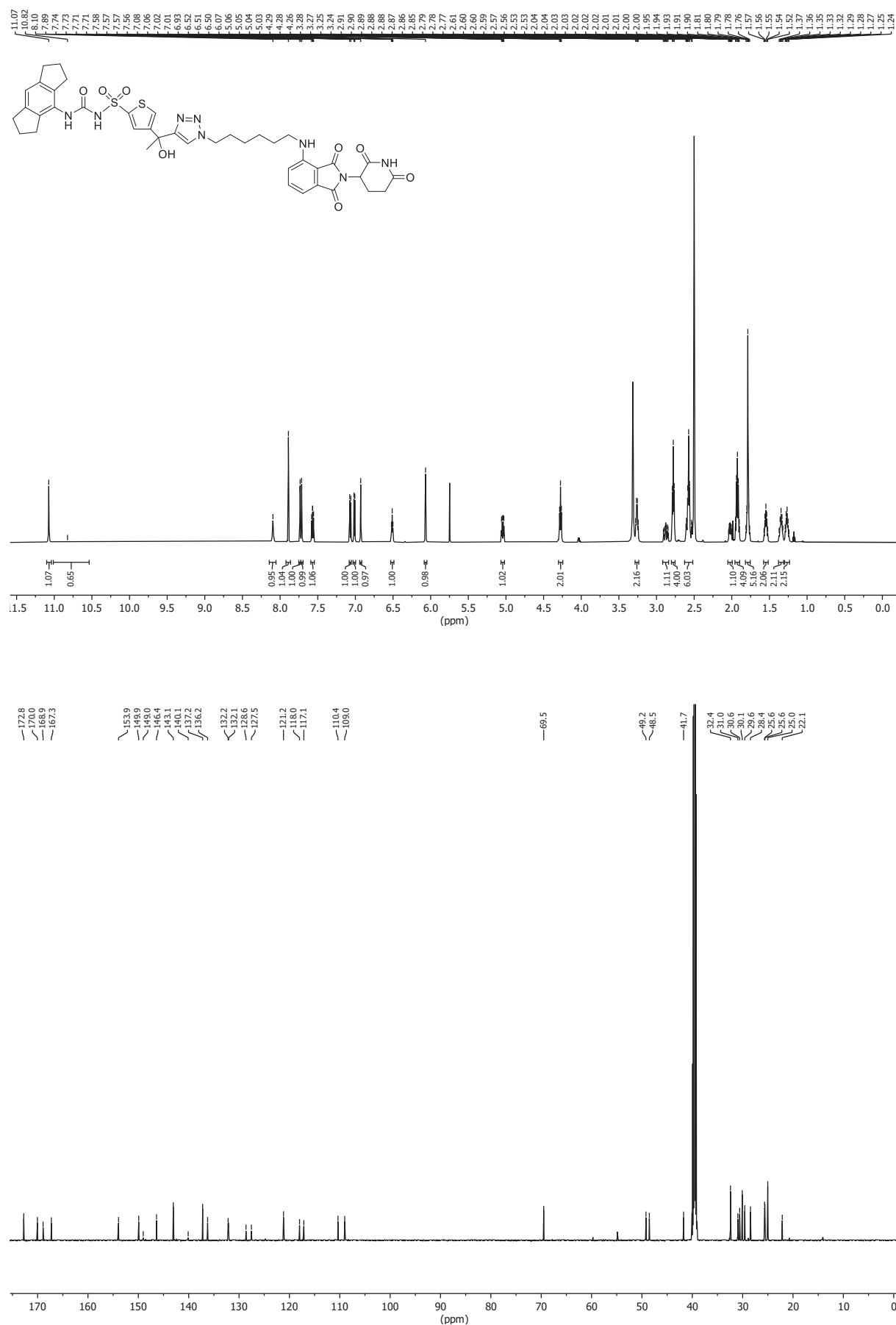
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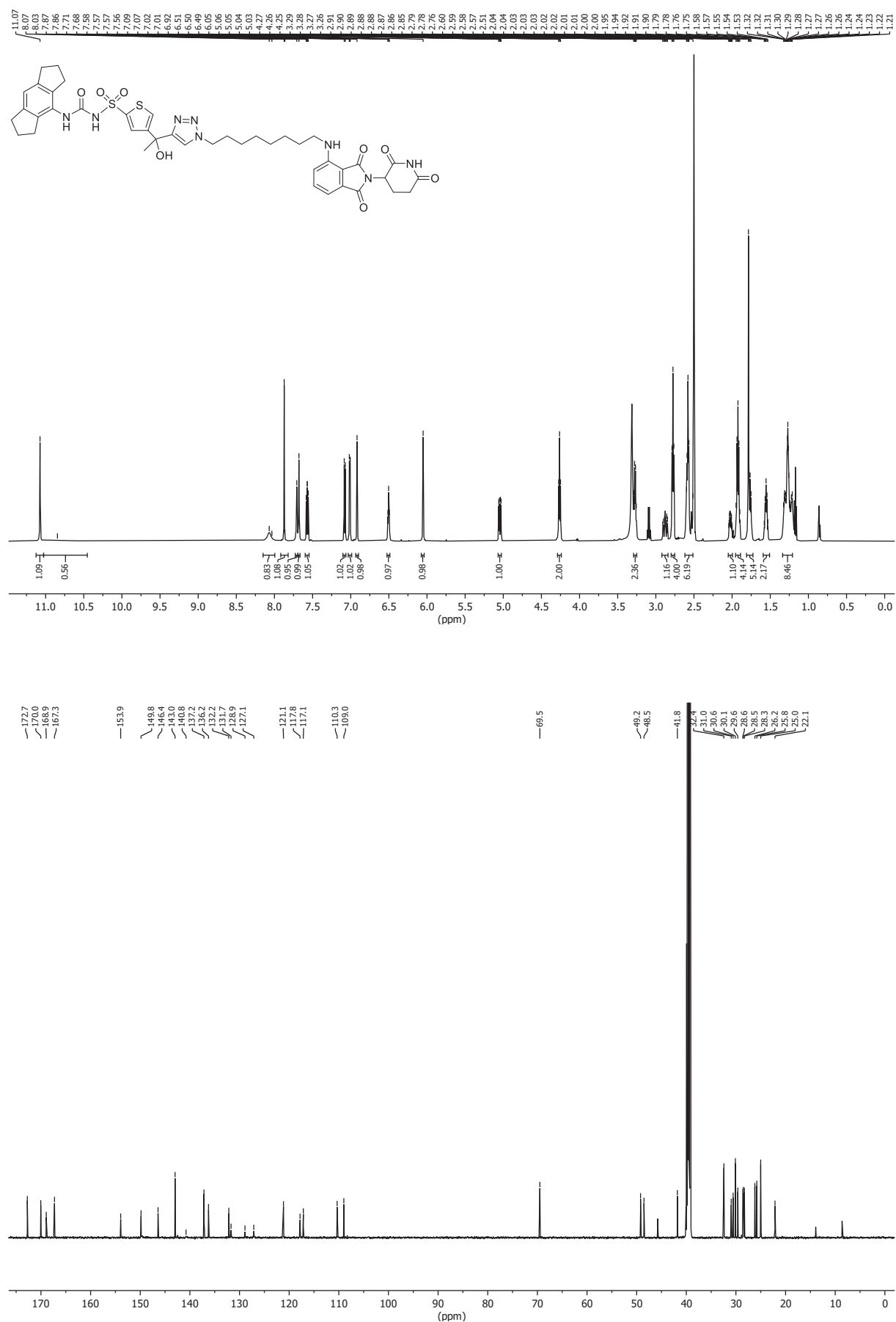
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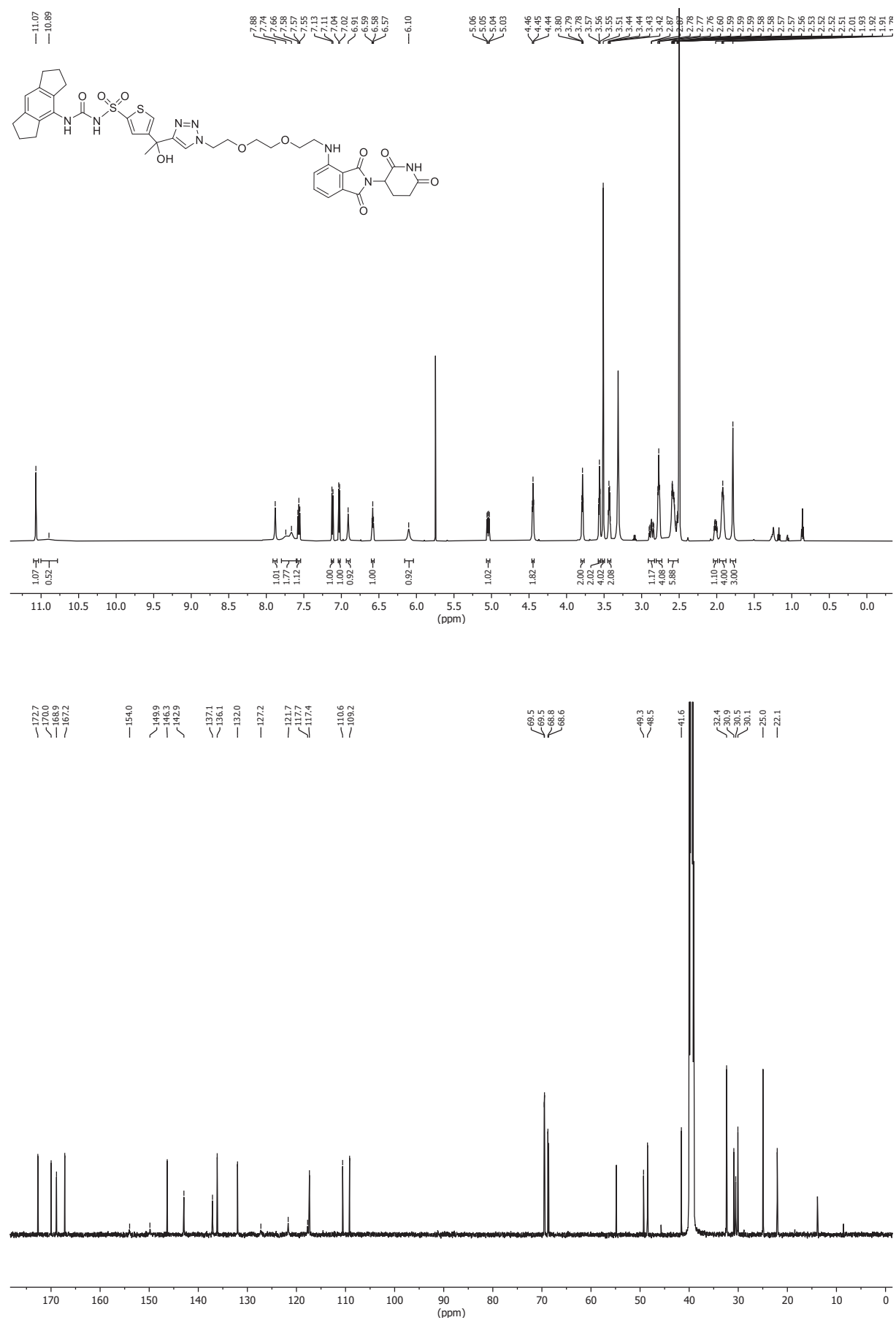
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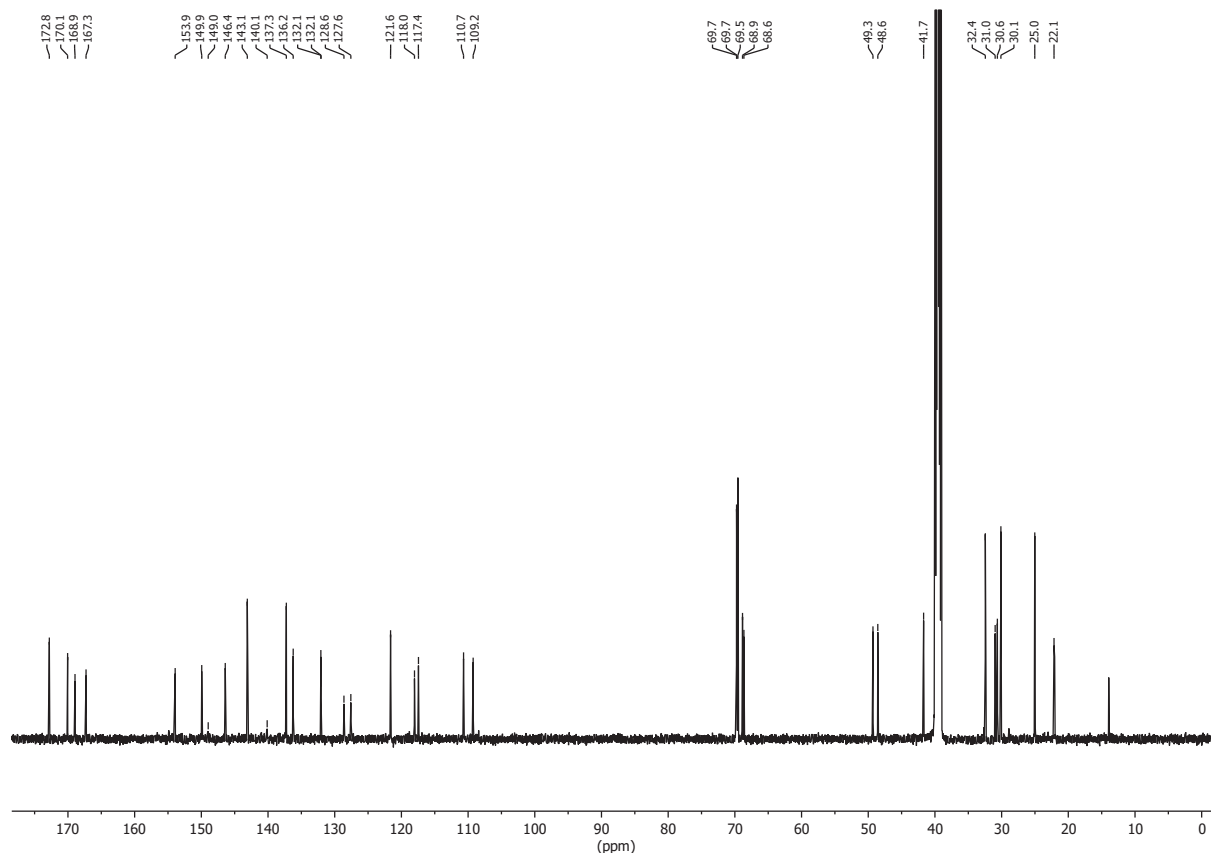
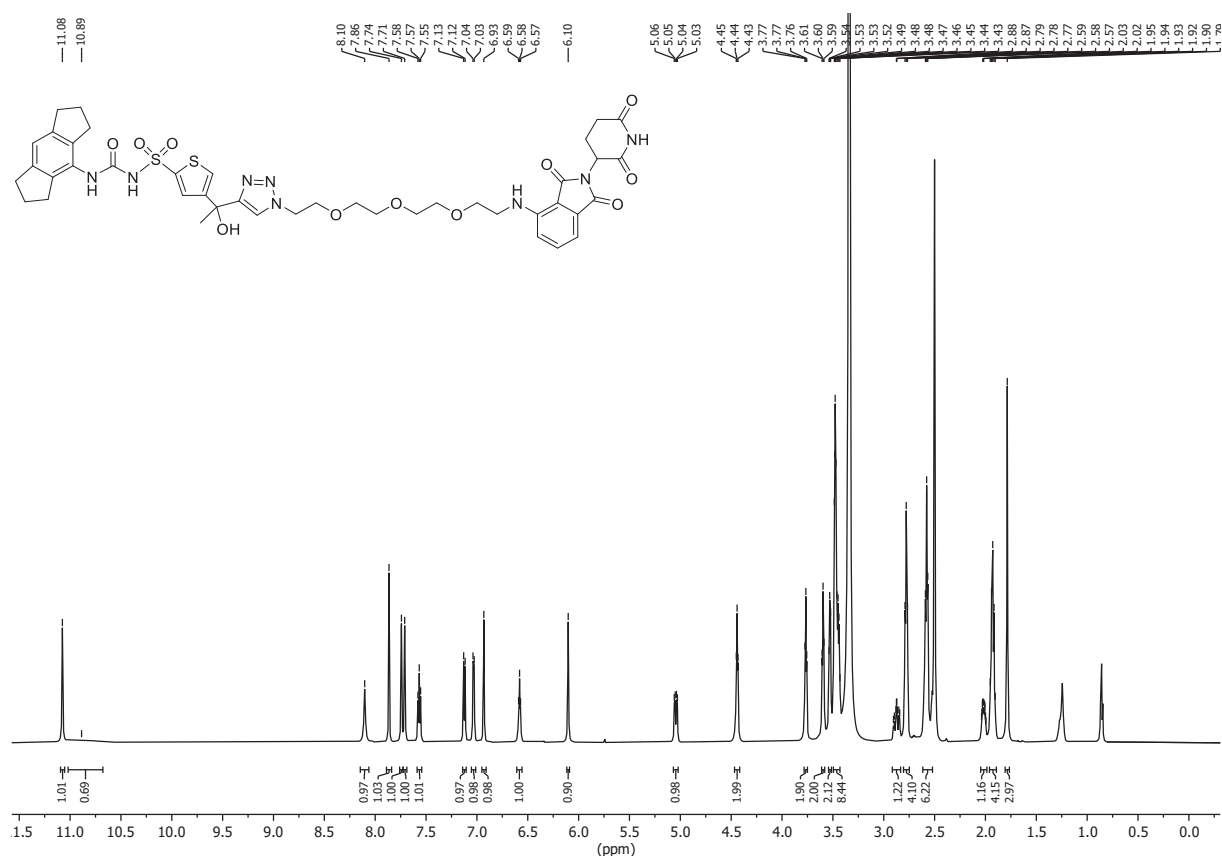
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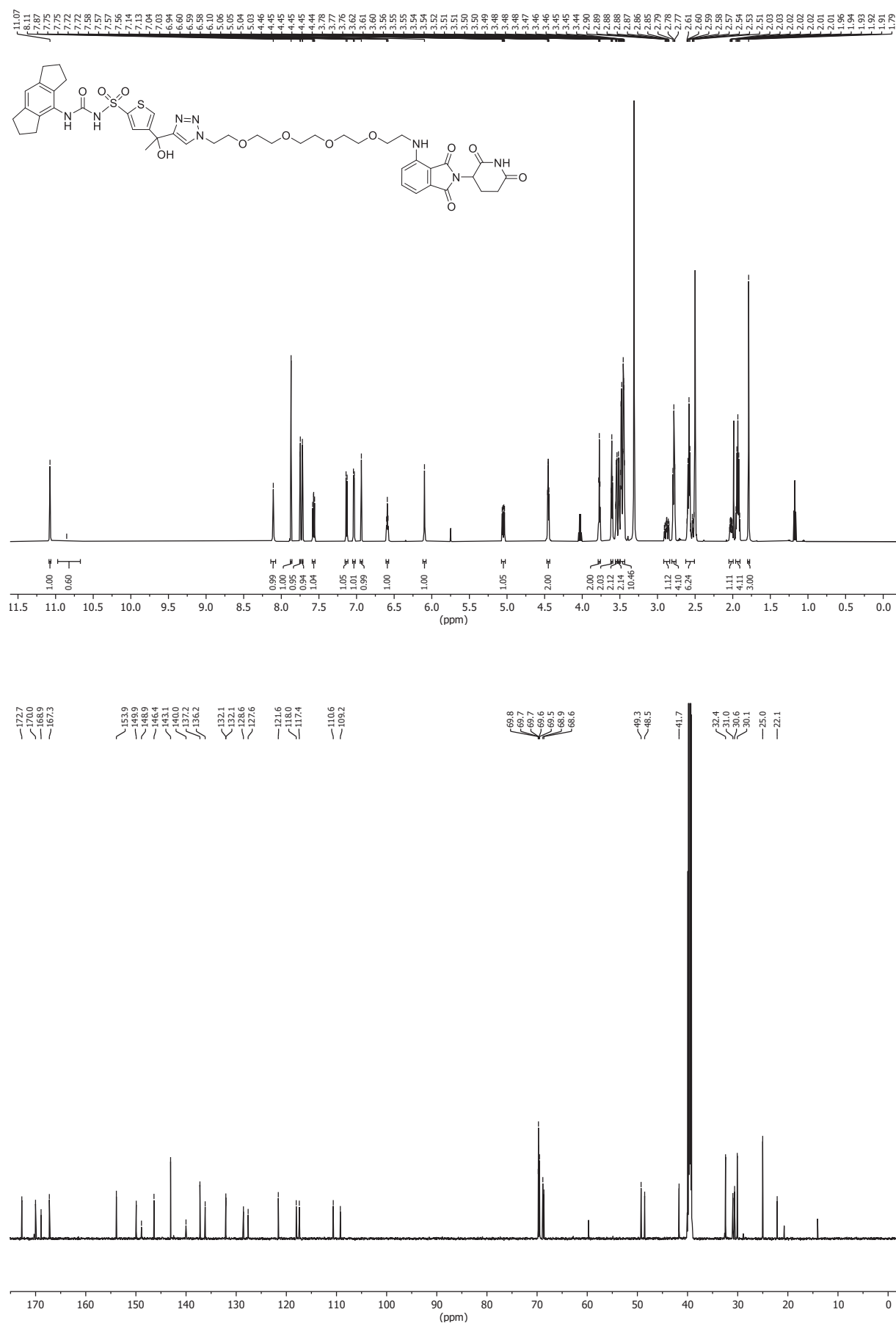
^1H and ^{13}C NMR spectra of compound **133**

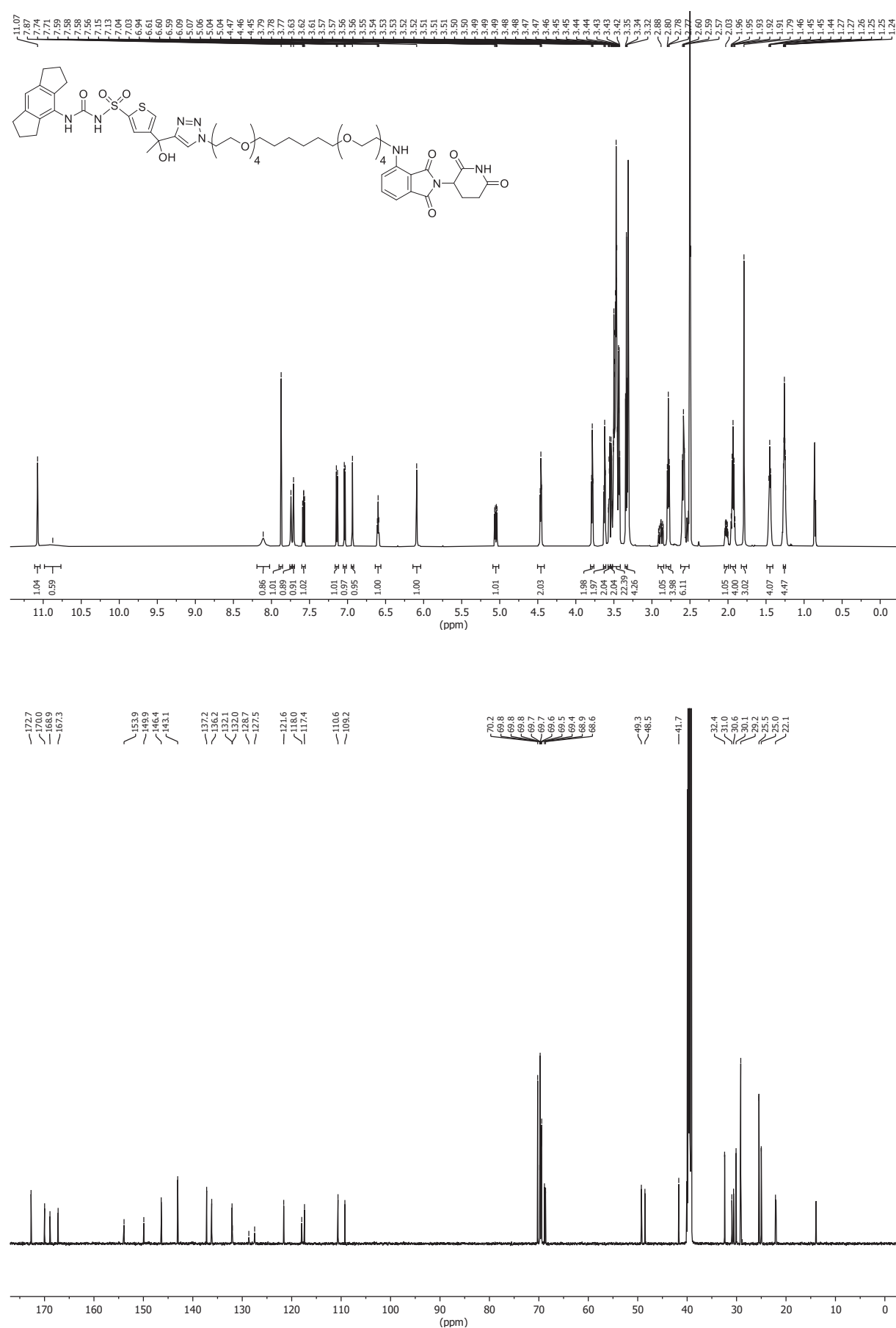
^1H and ^{13}C NMR spectra of compound **134**

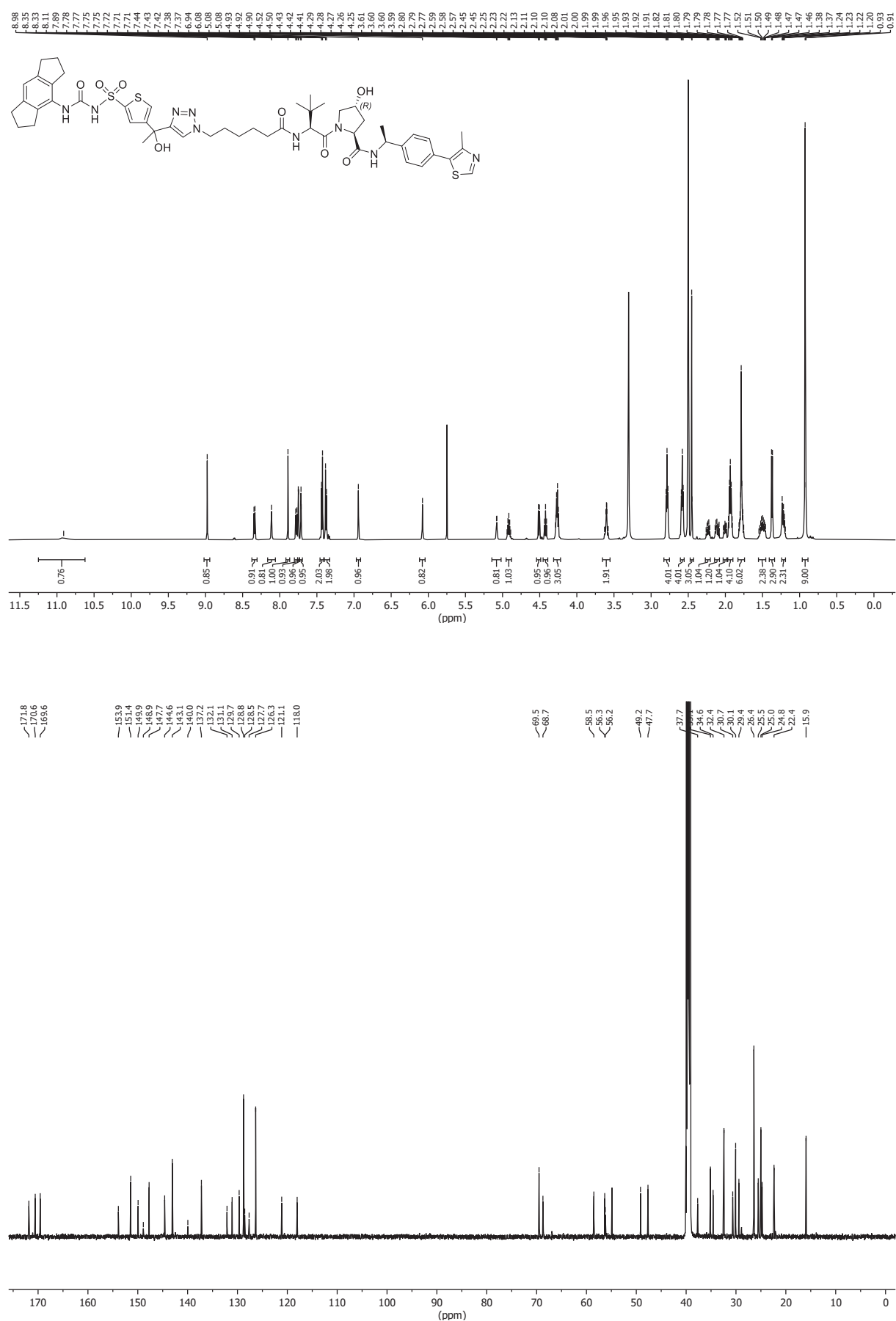
^1H and ^{13}C NMR spectra of compound **135**

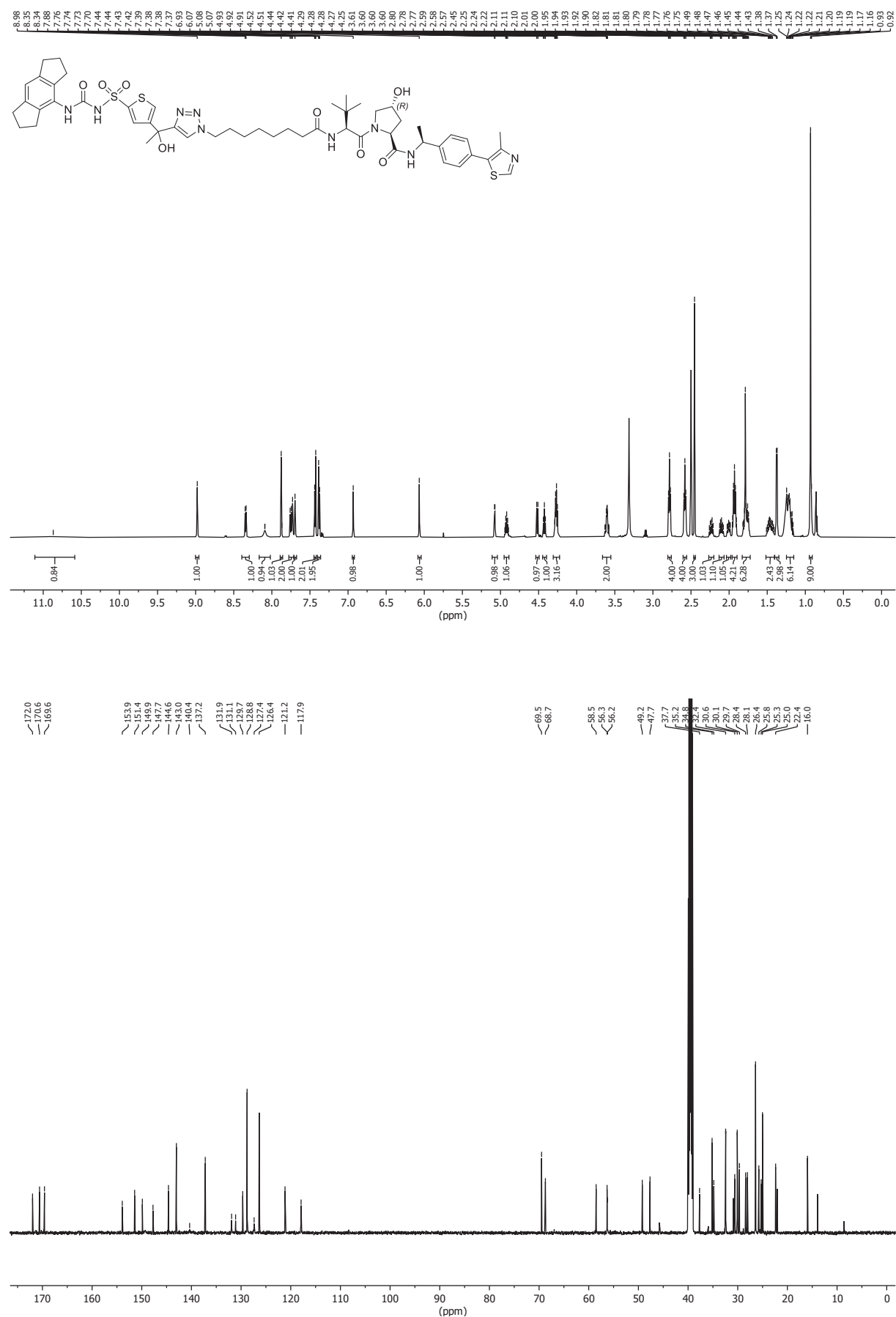
^1H and ^{13}C NMR spectra of compound **136**

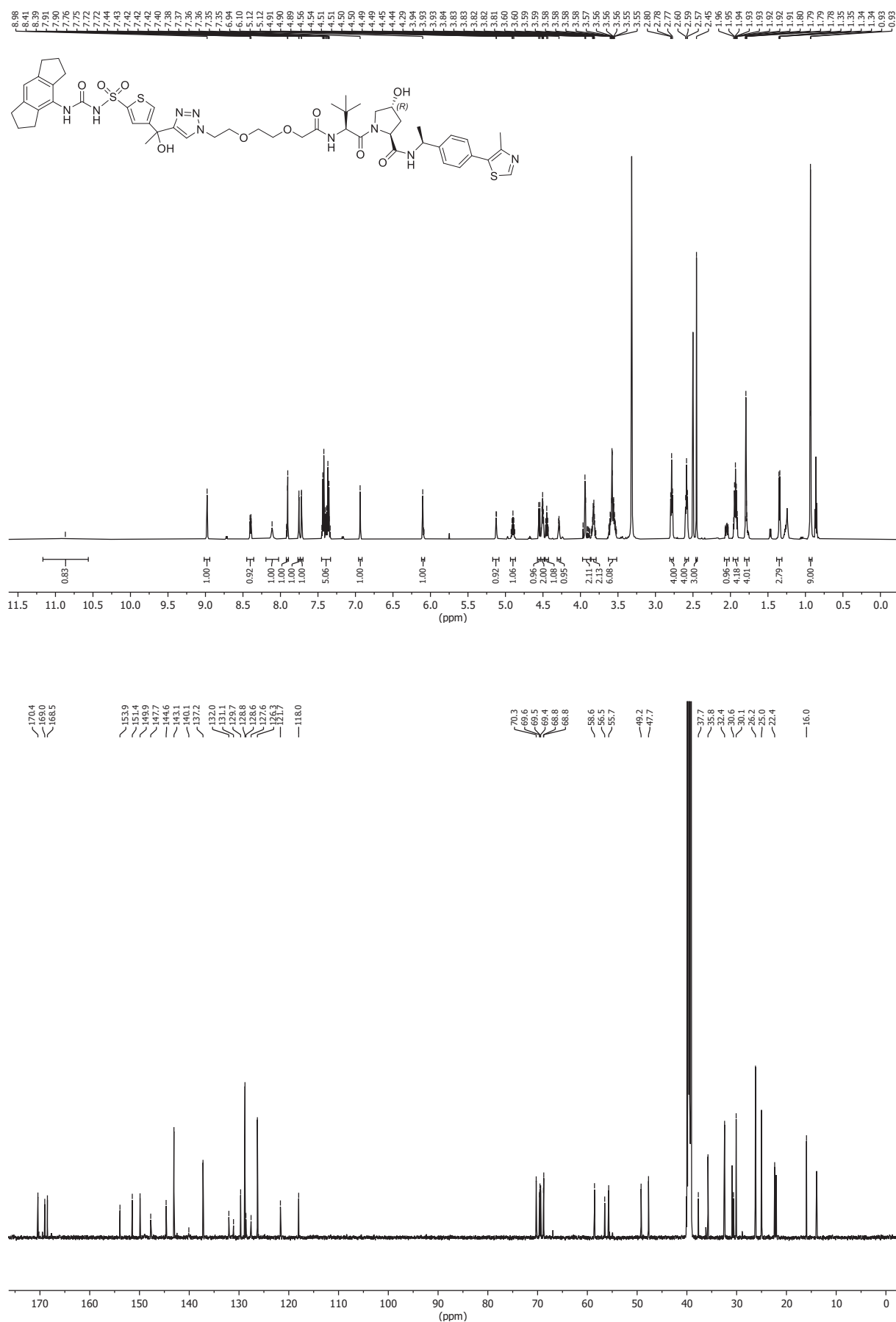
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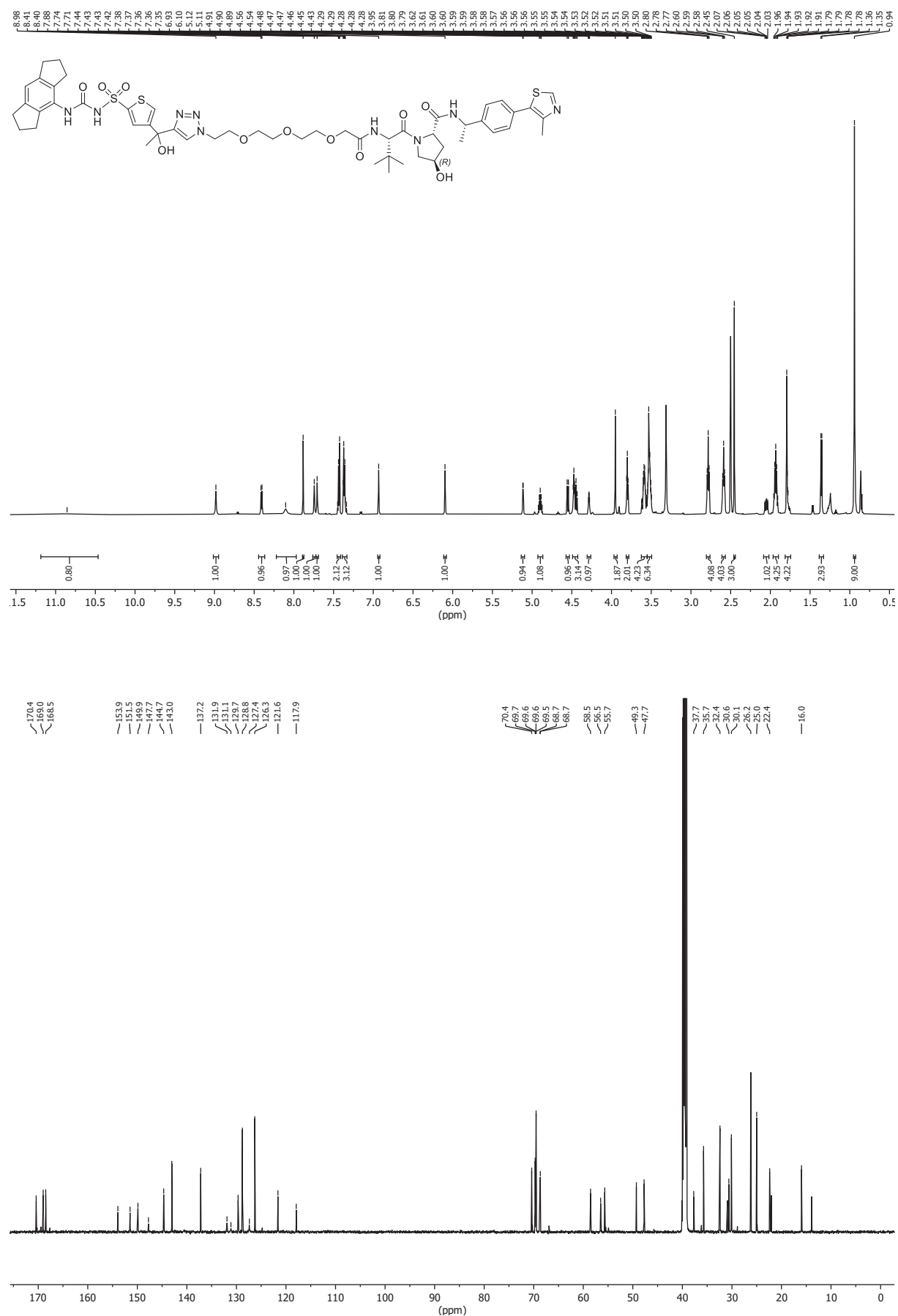
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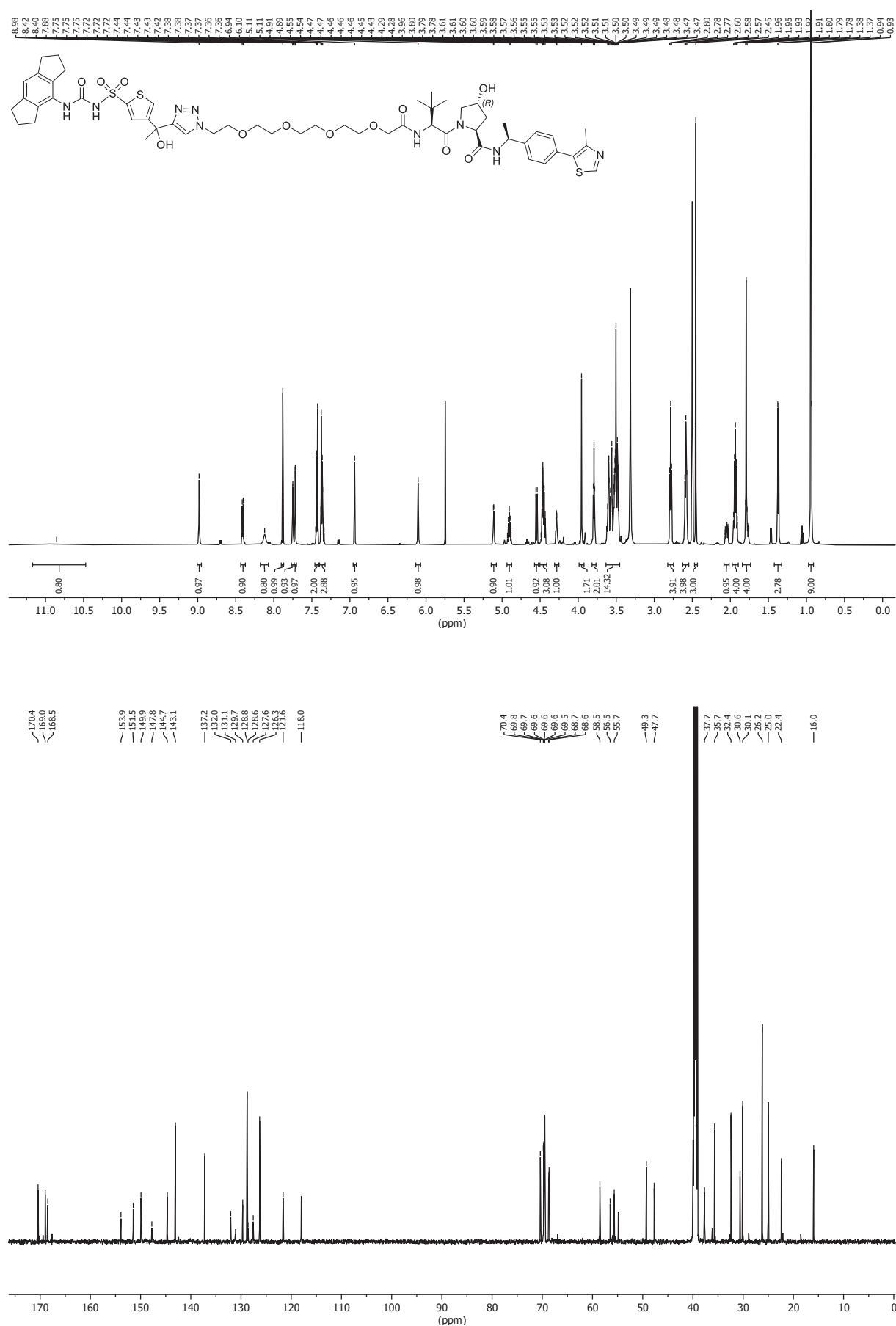
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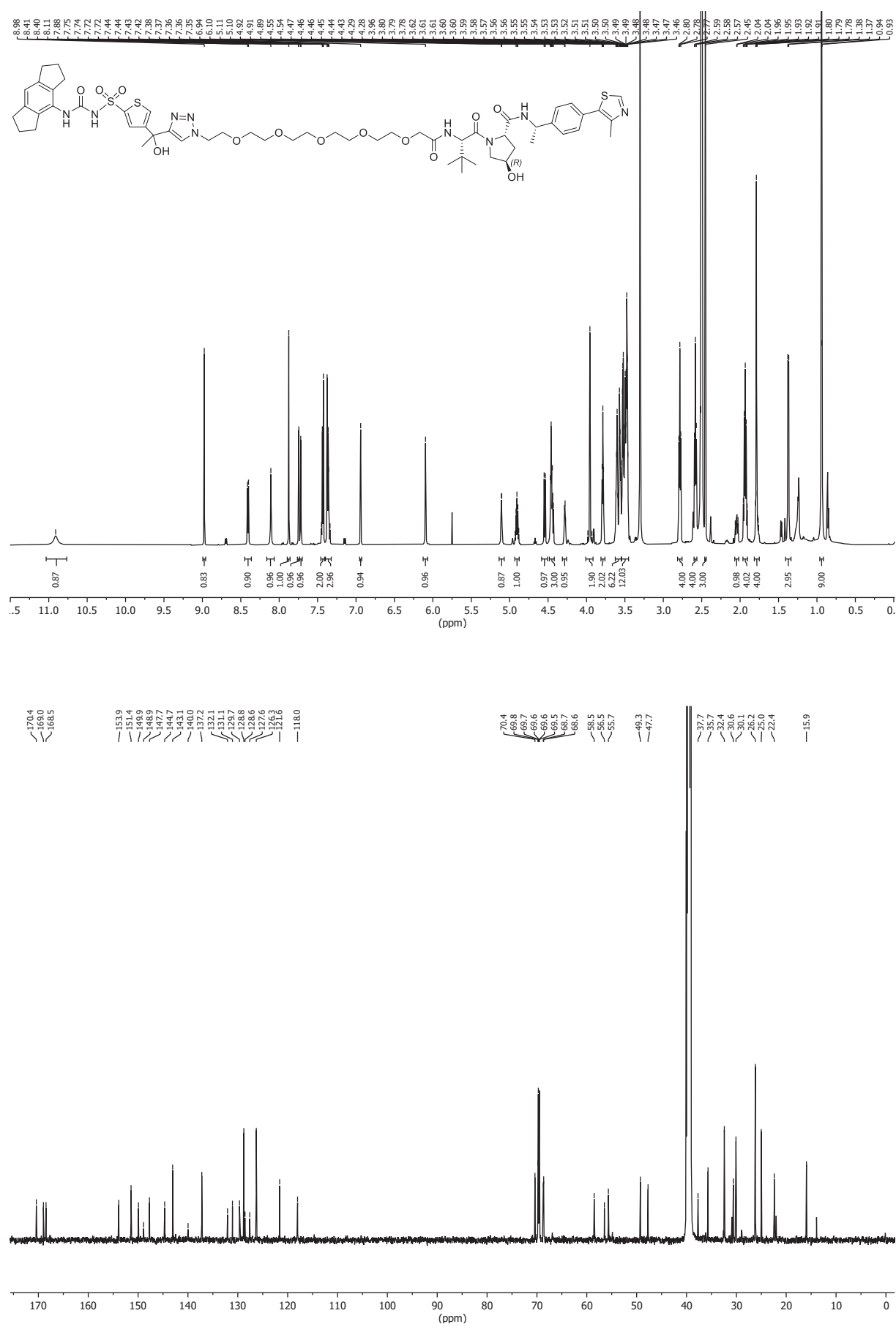
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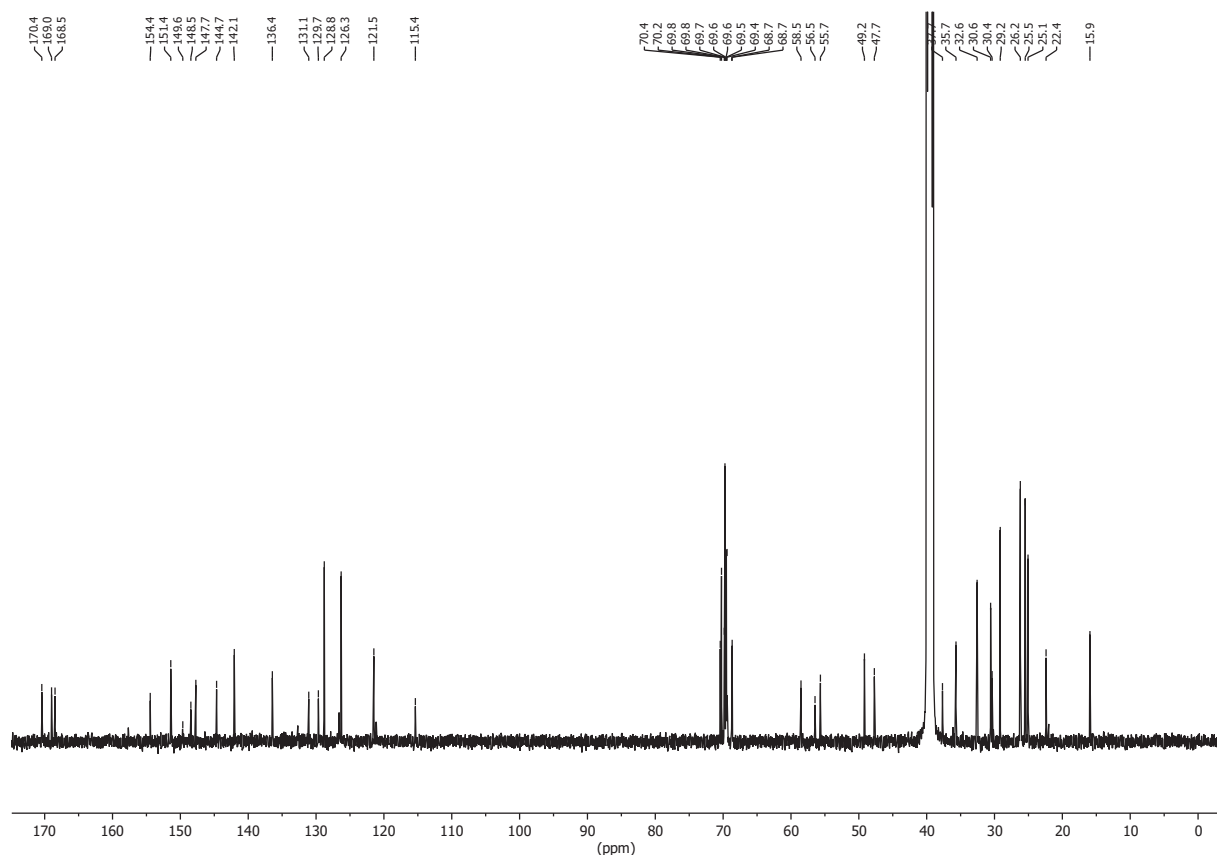
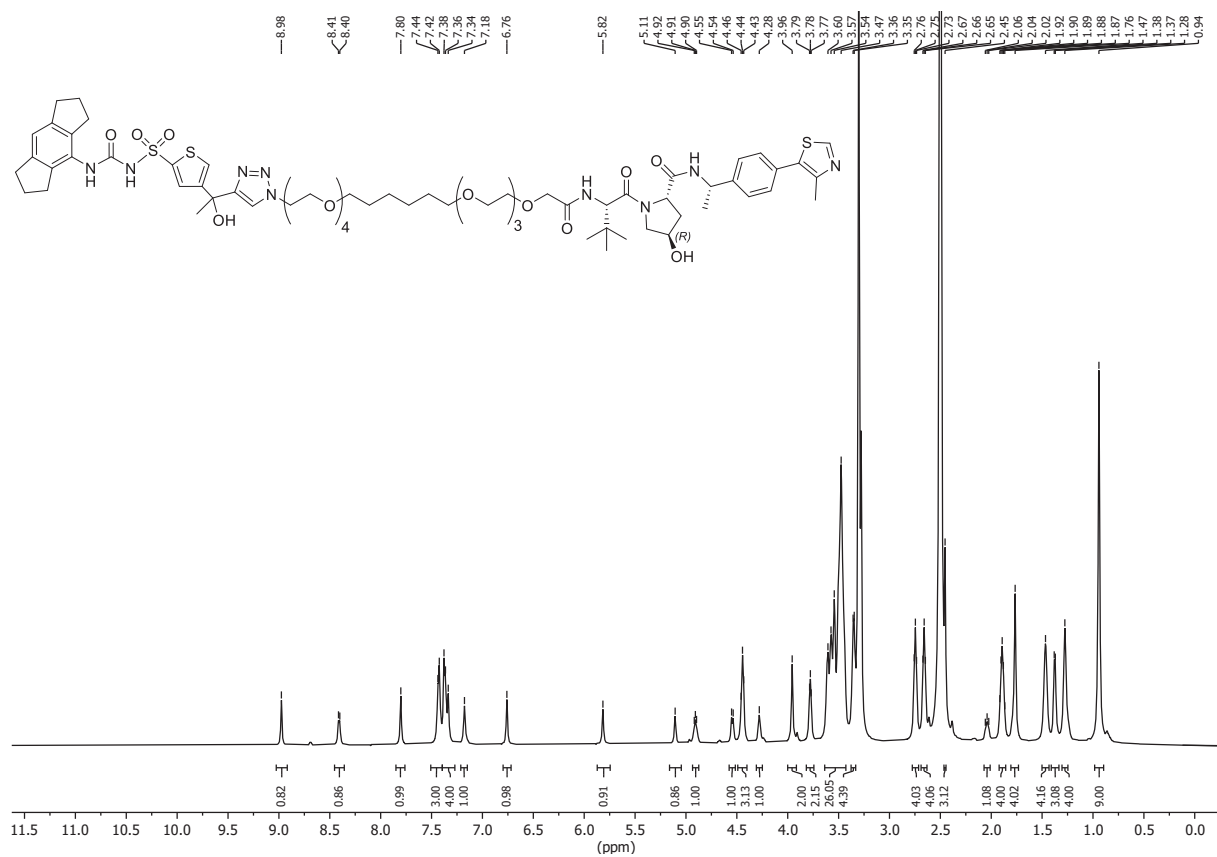
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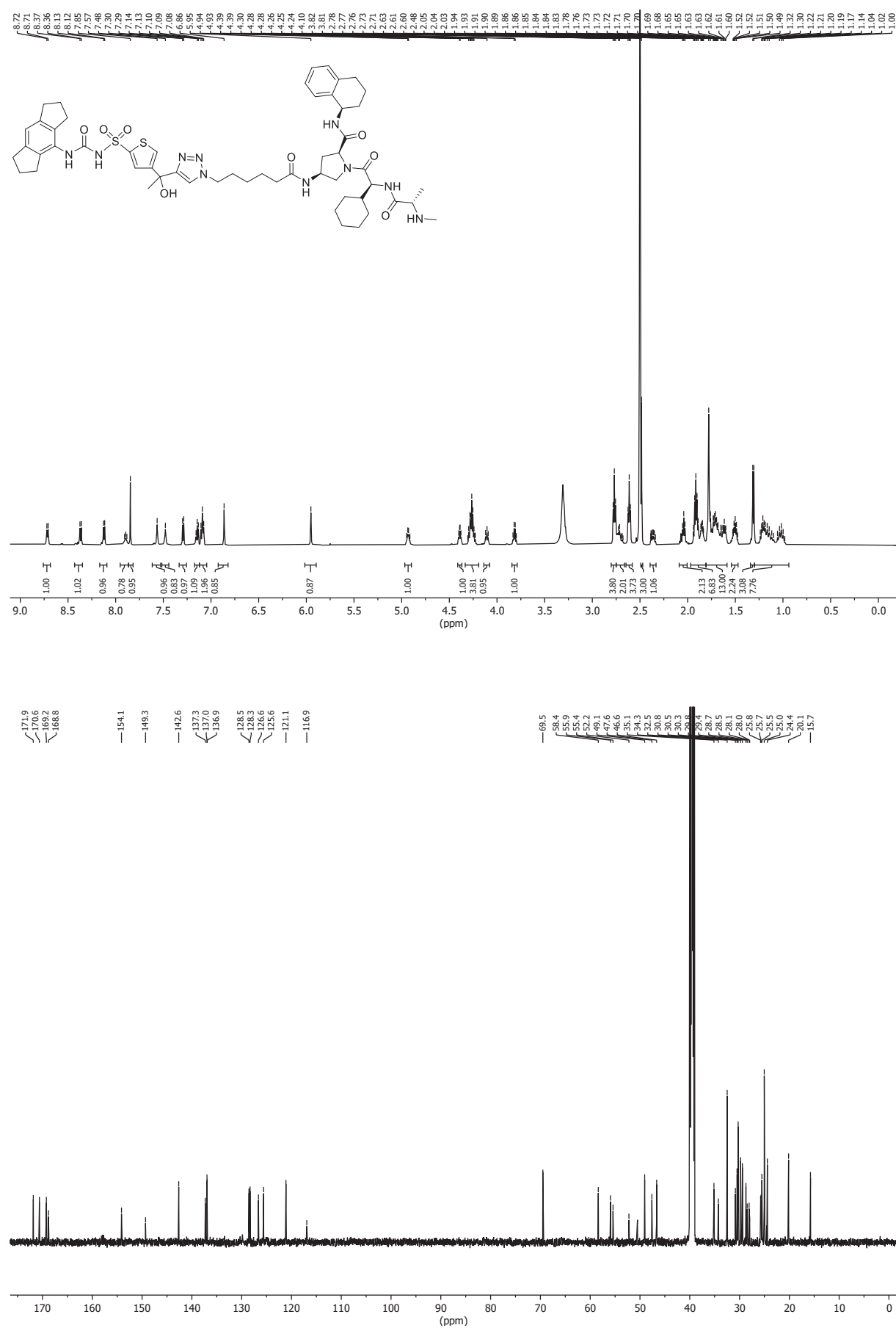
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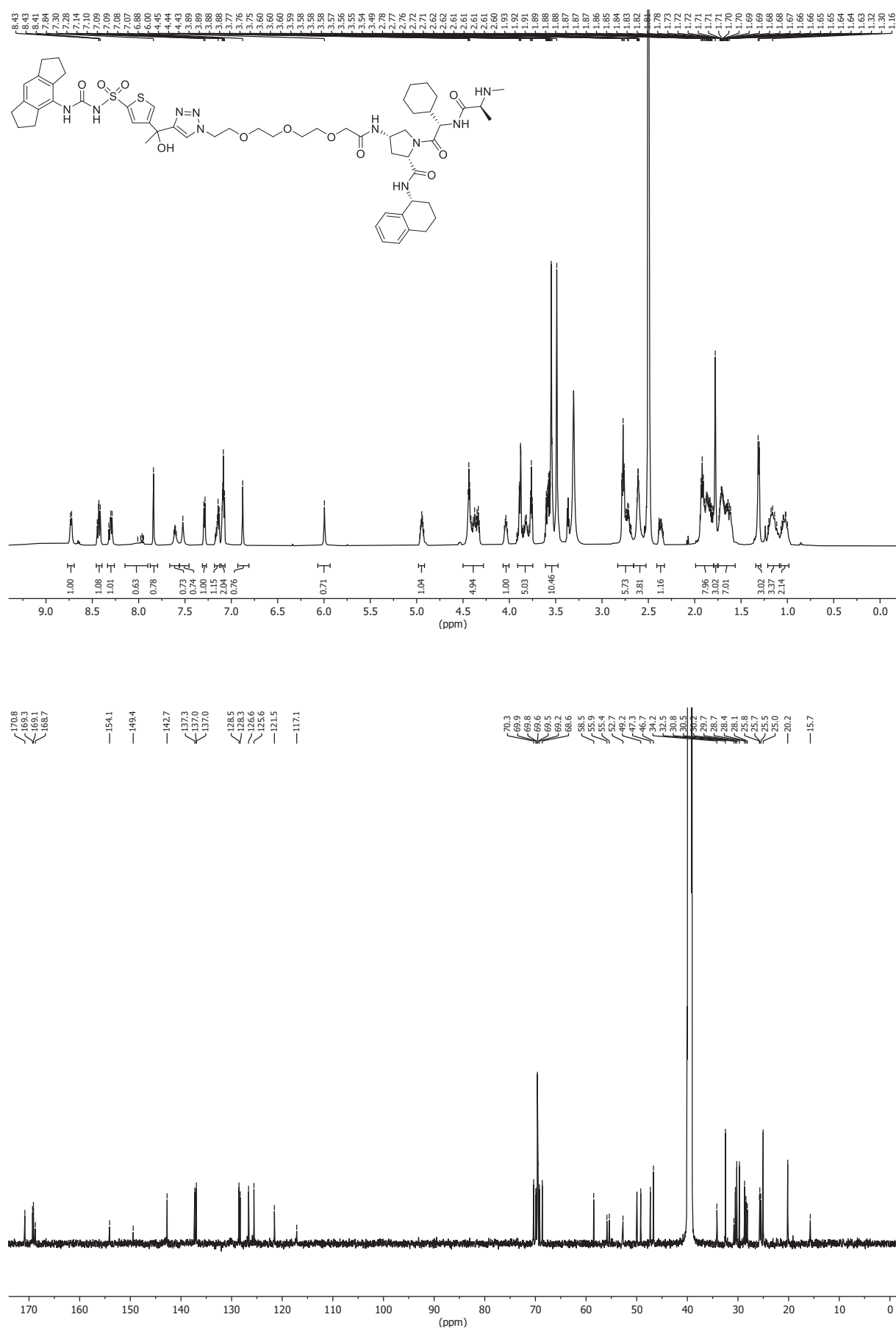
^1H and ^{13}C NMR spectra of compound **144**

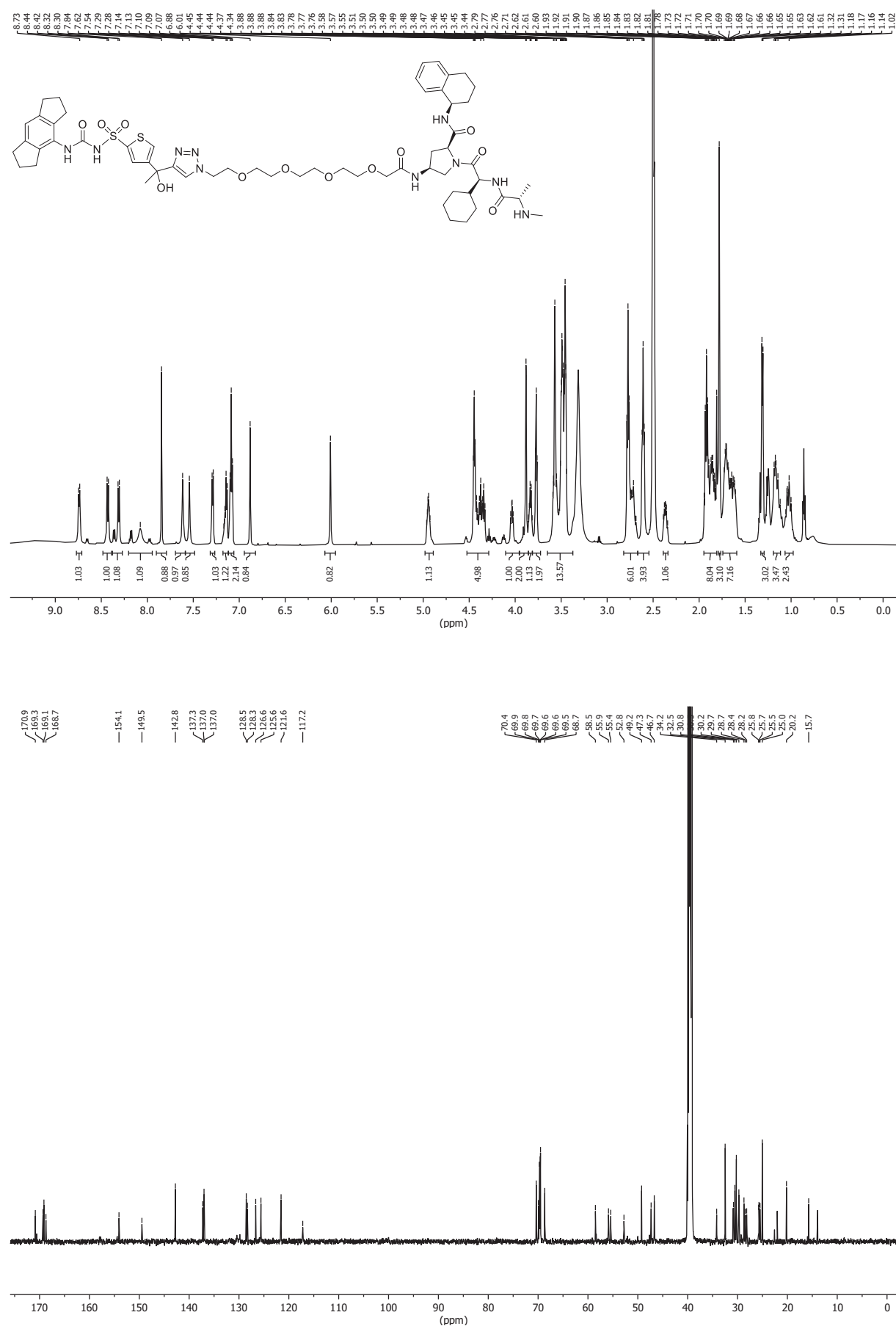
¹H and ¹³C NMR spectra of compound **145**

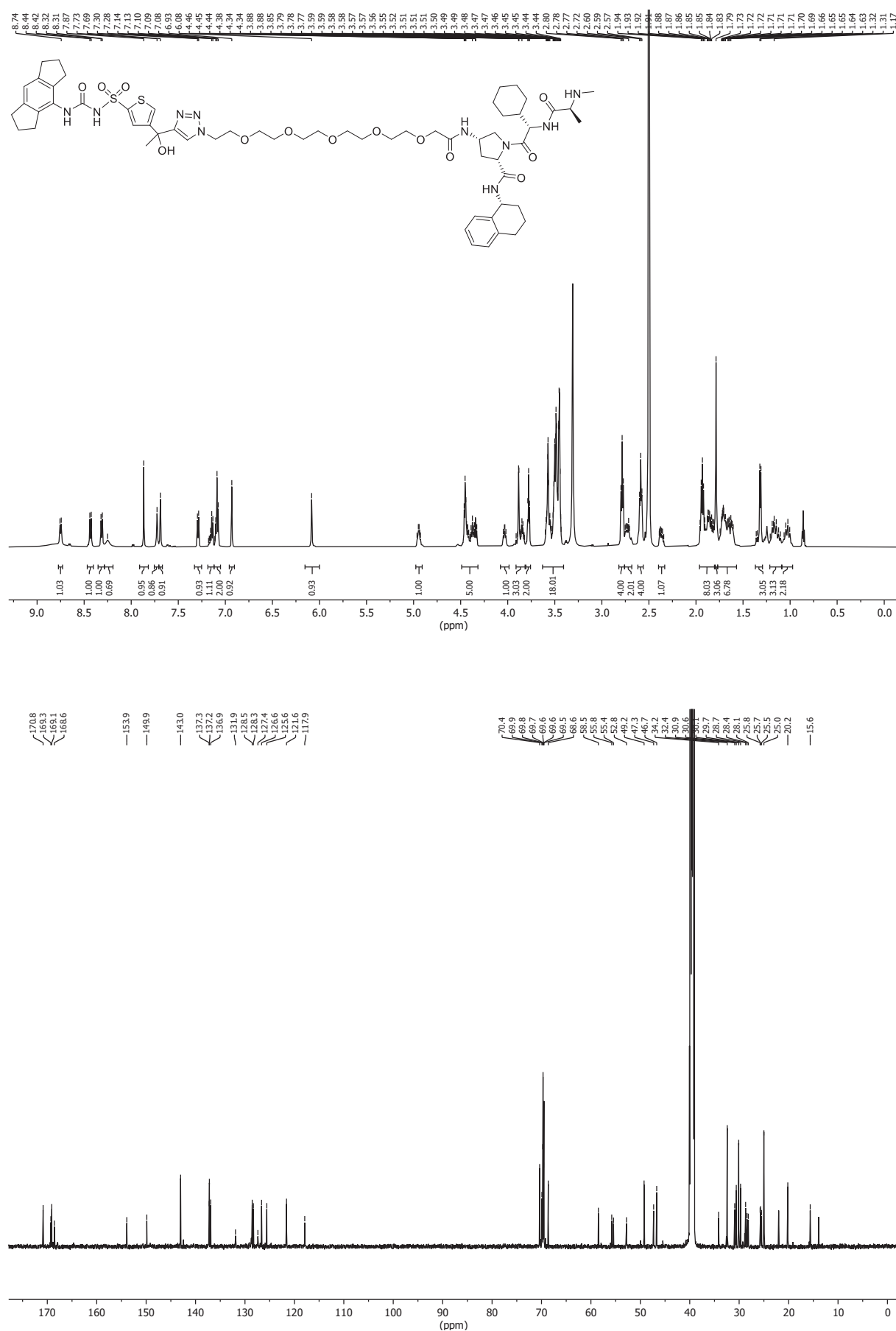
^1H and ^{13}C NMR spectra of compound **146**

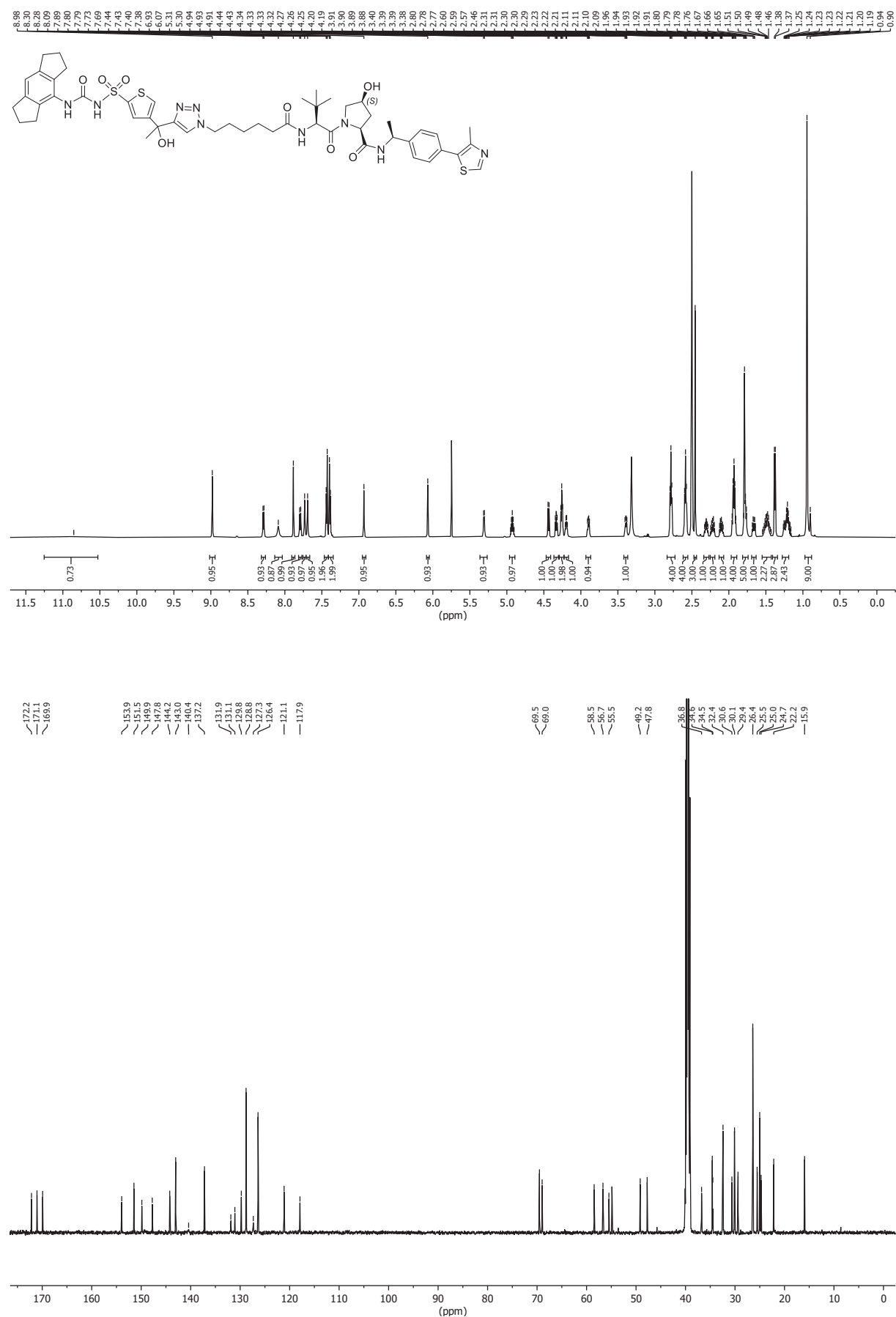
^1H and ^{13}C NMR spectra of compound **147**

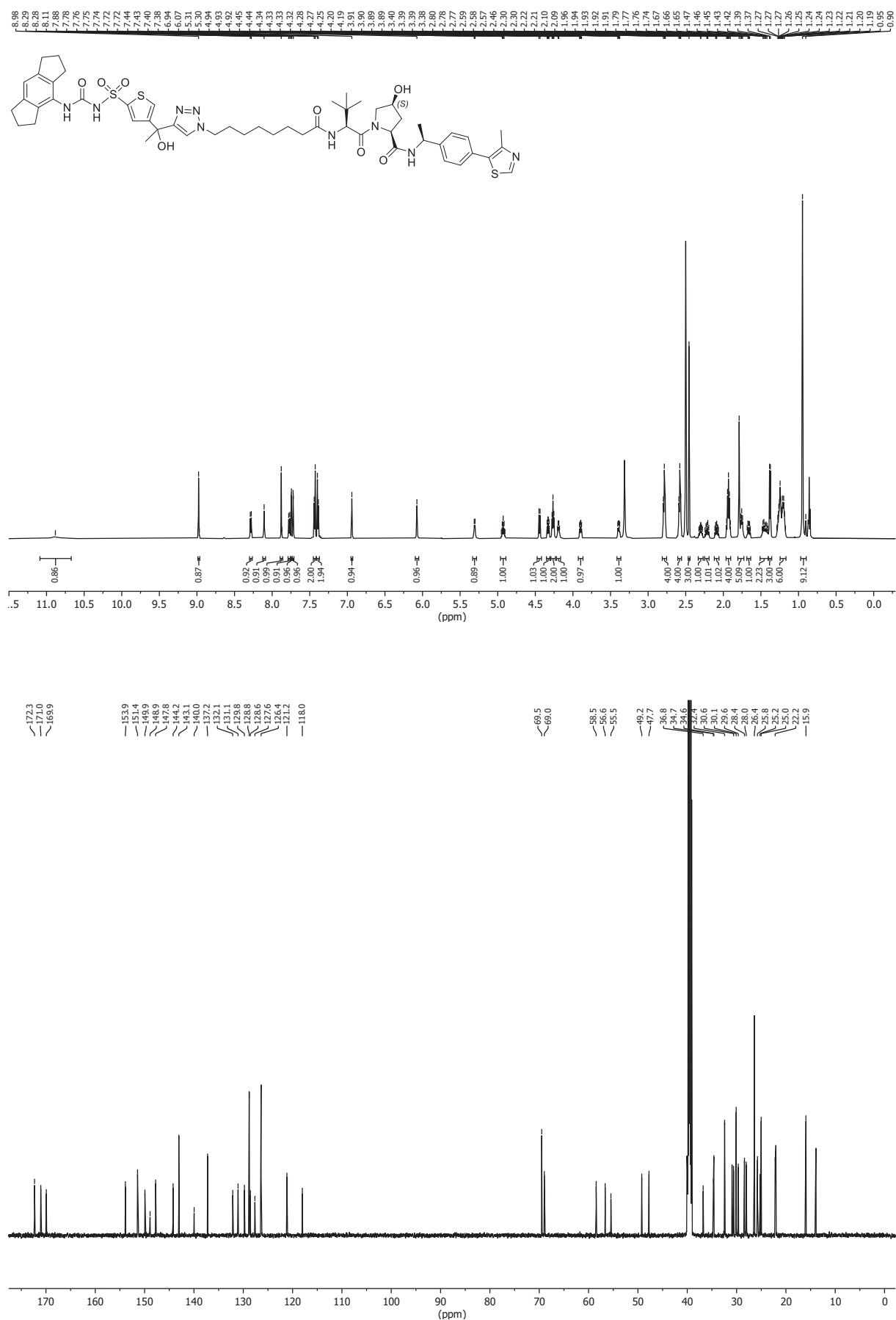
^1H and ^{13}C NMR spectra of compound **148**

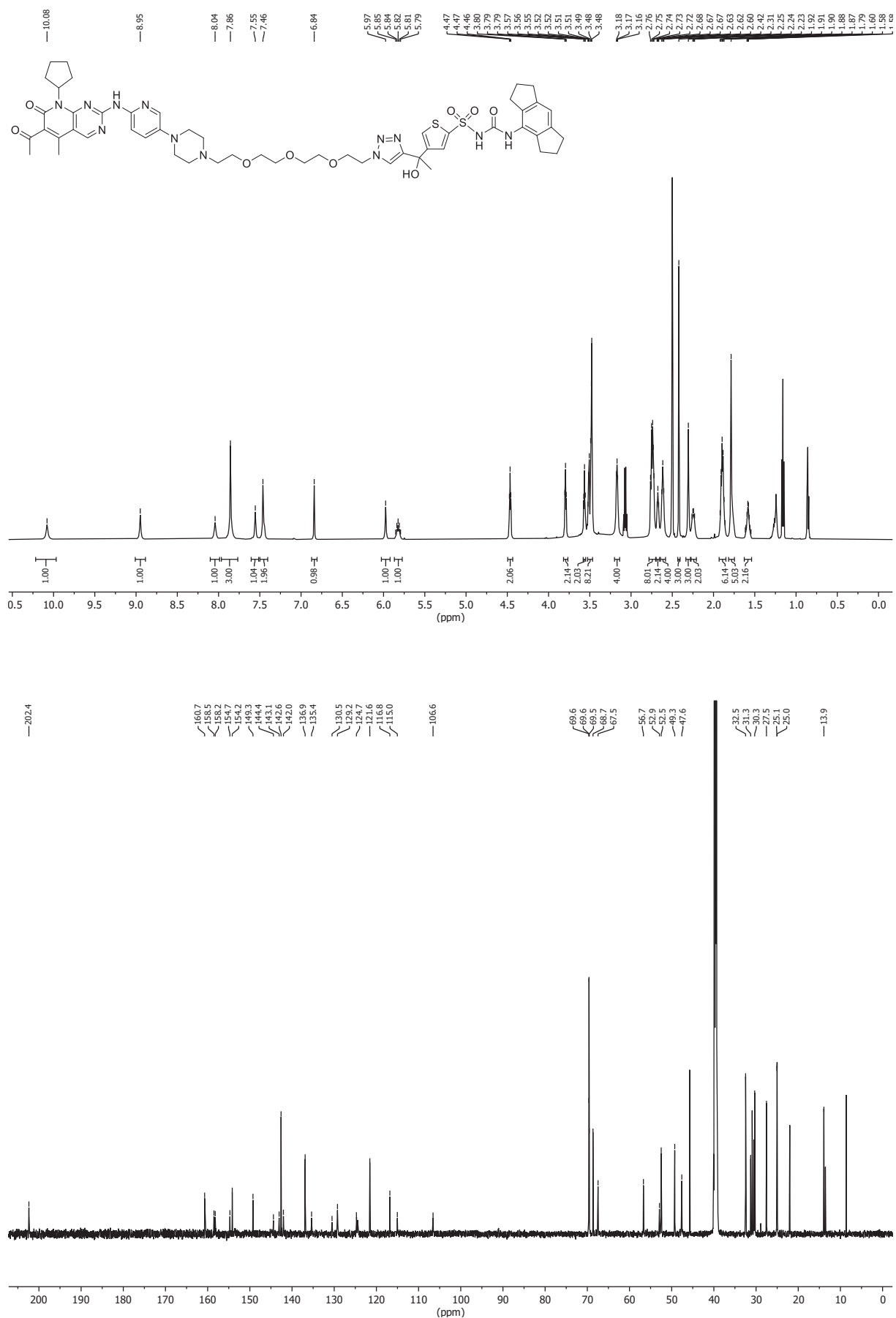
^1H and ^{13}C NMR spectra of compound **149**

^1H and ^{13}C NMR spectra of compound **150**

^1H and ^{13}C NMR spectra of compound **151**

^1H and ^{13}C NMR spectra of compound **155**

^1H and ^{13}C NMR spectra of compound **156**

^1H and ^{13}C NMR spectra of compound **163**

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